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**THE ROLE OF HUMAN PAPILLOMAVIRUS AND
OTHER RISK FACTORS IN MALIGNANT AND
PREMALIGNANT SKIN COMPLICATIONS
FOLLOWING HEART, HEART-LUNG AND LUNG
TRANSPLANTATION**

By

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Abstract

The role of human papillomavirus and other risk factors in malignant and premalignant skin complications following heart, heart-lung and lung transplantation.

Simal Soin

Addenbrooke's Hospital, Cambridge.

A side-effect of immunosuppression following renal transplantation is the development of skin infections and non-melanoma skin cancers which are associated with human papillomavirus (HPV) infection and sun exposure. Heart-lung transplant recipients are on average younger than renal allograft recipients and also receive a higher level of immunosuppressive therapy. To ascertain the incidence of HPV-associated cutaneous complications after heart and lung transplantation and the role of potential risk factors, we have investigated 397 of the surviving transplant recipients by means of questionnaire, review of medical records, and a proportion by interview and examination.

A high incidence of warts and non-melanoma skin cancers was found in heart-lung and heart transplant population studied in accordance with previous studies on renal transplant recipients. These skin complications of immunosuppression, especially warts, keratoses and cancers, were present in 249 patients (62.7%). Skin cancer was present in 17.6% of transplant recipients, all of whom were 5 or more years post-transplant. The squamous:basal cell carcinoma ratio was 8:1. HPV DNA was detected in 13 of 31 SCC samples analysed. In 6 cases, the HPV was HPV 20. 86% of the patients with skin cancers had high levels of lifetime occupational or social sun exposure.

Sun exposure, male gender and increasing graft survival time have emerged as major risk factors with minor influences from age and level of immunosuppression. No HLA associations were found in the patients with malignancy in contrast to observations in kidney transplant patients.

Warts were found in 33.8% of patients, with a significantly higher incidence in males than in females, but were not influenced by levels of sun exposure.

A thesis submitted for the degree of Master of Philosophy at
the Open University, U.K.

Division of Virology
Department of Pathology
University of Cambridge

I hereby acknowledge that the thesis represents my own work, except where duly acknowledged in the text. The contents have not been submitted for a degree at this or any other university.

Simal Soin

To
Avi
For everything

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Abbreviations

ALG	Antilymphocytic globulin
ATG	Antithymocytic globulin
BCC	Basal Cell Carcinoma
bp	base pair(s)
DEAE	Diethylaminoethyl
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid.
dNTP	Deoxynucleotide
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylene diamine tetra acetic acid
EV	Epidermodysplasia verruciformis
GC	Guanine Cytosine
HLA	Human leucocytic antigen
HPV	Human papillomavirus
IPTG	isopropyl b-D-thiogalactopyranoside
MgCl ₂	Magnesium chloride
NaOAc	Sodium acetate
OKT3	anti CD3 monoclonal antibody
ORF	Open reading frame
PBS	Phosphate buffered saline
RNase	Ribonuclease
rpm	revolutions per minute
SCC	Squamous Cell Carcinoma
SDS	Sodium dodecylsulphate
TBE	Tris-borate-EDTA
TE	Tris EDTA
Tris	tris (hydroxy methyl) methylamine
2TY	Bacto-tryptone

x-gal

5-Bromo-4 chloro-3 indolyl D-galactoside

Chapter 1

Background

1.1 Introduction

1.1.1 Skin complications in transplant patients

- a. Incidence and spectrum
- b. Risk Factors
- c. Prevention and Treatment options

1.1.2 Human Papillomaviruses

1.2 Aims of the Study

CHAPTER 1

Background

1.1.1 Introduction

Organ transplant recipients experience a very high frequency of skin complications (1). In fact skin disease is the most frequent untoward effect experienced by most long-term transplant patients. Immunosuppression, human papillomavirus infection and sunlight exposure are thought to be the main risk factors responsible. To a smaller extent, HLA type and mismatch, age, sex and ethnic variations may play a part. The commonest long term skin complications are tumours, including both benign lesions such as warts and solar keratoses and also skin cancers such as basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). Less common tumours include keratocanthoma, Bowen's disease, Kaposi's sarcoma, porokeratosis and anogenital malignancies such as cervical, vulval and anal cancers.

The short-term complications include infective lesions which may be bacterial, viral, fungal or protozoal in origin, and non-infective lesions caused specifically as a result of immunosuppressive therapy such as xerosis, cushingoid features, hypertrichosis, striae, facial erythema and friable skin (19). Short-term complications occur during the period immediately and few months posttransplant, mainly as a result of the reduced immunity, caused by high doses of immunosuppressants to prevent rejection and to ensure a smooth

transition into the future. Long term complications are those which occur after the graft survival has exceeded five years. Post-transplant, long-term skin tumours are a matter of concern in the longer term due to the following important differences from lesions occurring in the non-transplant population:

- a. Their incidence is considerably higher (37, 45, 69).
- b. Skin cancers occur 15-20 years earlier than their counterparts in a normal population (65, 71).
- c. The spectrum of malignant and premalignant skin lesions in the posttransplant immunosuppressed is quite different (28).
- d. Natural history of skin cancers in transplant recipients is more aggressive (7, 65).
- e. They cause considerable morbidity since they can be resistant to treatment, and occur repeatedly.
- f. Pre-malignant conditions like keratoses undergo accelerated progression to in situ invasive squamous cell carcinoma (50).

The data mentioned here are mainly derived from renal transplant recipients as they are the most extensively studied group. While there is some data in the literature relating to heart transplant patients, the risk and causative factors have not been clearly defined. One would expect them to have similar complications.

Recipients of heart, heart-lung and lung transplants form a rapidly growing cohort of patients. They generally receive higher doses of immunosuppressive drugs than renal transplant recipients and although the age range of heart transplants is comparable to the renal transplant recipients (23-58 years) heart-lung transplant recipients are generally much younger (20-40 years). Most of them are in their twenties and early thirties. Heart transplant recipients are documented to suffer with similar types of skin problems to renal

transplant recipients such as warts, skin cancers, solar keratoses (25, 41) but the series reported to date are small. There are no published studies documenting the risk and spectrum of skin disease in heart-lung transplant recipients. This project involved a detailed study of thoracic transplant recipients at Papworth Hospital.

a Incidence and spectrum of post-transplant skin problems

Skin cancer

The incidence of post-transplant skin complications is greatest in areas with high intensity of sun exposure with rates increasing with time after transplantation. The incidence of post-transplant skin cancer in Australia has been reported to rise from 7% 1 year after transplantation to 70% 20 years post-transplant (14). Even in regions of temperate climate skin cancers are now a significant problem. European studies show a risk of development of SCC of 10%, 10 years post-transplant increasing to 40% at 20 years (22, 39, 49). The risk is greatest in Caucasians though even in white-skinned populations, racial groups such as the Celts have a greater susceptibility to non-melanoma skin cancers (44). In the general population, basal cell carcinomas outnumber squamous cell carcinomas by 5-8:1. In transplant patients, the incidence of SCCs is increased 20 to 50 fold and BCCs is increased 10 fold over the general population (39). Hence, the ratio of the two cancers is reversed, with SCC : BCC varying from 1.8-3.6:1 (25, 37 39, 65). SCCs and BCCs occur on different sites, with SCCs occurring mostly on sun exposed sites such as the face and the back of the hands and BCCs also on the face, but also on the chest and back not on the backs of the hands. This probably demonstrates a difference in aetiology (39). A report published in 1991 from The Cincinnati Transplant Tumour Registry on

over 5000 post-transplant cancers showed that cancers in recipients of non renal organs had a different pattern to that in recipients of renal organs. The commonest malignancy in non renal recipients was lymphoma whereas in renal graft recipients it was skin cancer (87). This discrepancy could have been due to the small numbers of non renal patients seen, since subsequent studies have shown a high incidence of skin cancer even in heart transplant recipients (25, 41). The incidence of lymphoma and Kaposi's sarcoma is higher in non-renal transplants than renal transplant recipients because of the intense immunosuppressive therapy they receive to reverse rejection, whereas renal transplants would revert to dialysis (65).

Premalignant lesions and warts

Warts can reach an incidence of 90% in patients 5-10 years post-transplant (45, 69). The incidence of dysplastic epidermal keratoses in a prospective study of kidney transplant patients at a mean period of 2 years post-transplant, reached 14% (30). There is however evidence of accelerated progression of these lesions to malignancy (50). Factors, which could be involved in the transition of a premalignant lesion to a carcinoma, are activation of oncogene(s), inactivation of tumour suppressor gene(s), integration of HPV DNA into the host and failing immunity.

b. Risk factors

Immunosuppression

Some studies (3, 47) report that only the duration of immunosuppressive therapy is significant for the development of skin complications but others report that the level is also important since

reduction in the dose has been associated with a concomitant reduction in the multiplicity and rapidity of recurrence of skin cancers (11, 62). It is not known whether there is a certain threshold level of immunosuppression above which the incidence of skin complications ceases to increase any further. Comparisons between groups of transplant patients on different levels of immunosuppression would be needed to clarify this and hence, further studies are needed to establish the relative importance of dose and duration in this context. A recent Italian study (43) evaluated the first rejection score which was used as an indirect marker of the level of immunosuppression and which appears to be a better predictor of patients at risk for squamous cell carcinoma than cumulative immunosuppression. They concluded that both the level and duration of immunosuppression are relevant risk factors in the occurrence of SCCs. The duration of immunosuppressive therapy seems to be an important factor in acquiring SCCs but not BCCs (11) and BCCs tend to occur earlier in the course of immunosuppressive therapy as compared with SCCs (29). The number of episodes of rejection requiring short sharp bursts of immunosuppressive therapy does not seem to have any effect on the subsequent development of neoplasia (48). The occurrence of skin cancer and anogenital cancers is reduced according to some studies when cyclosporin based regimes are used, as opposed to those comprising azathioprine and steroids, even when different follow up times were considered (11, 64, 65). But other studies showed no difference (12, 14, 36) probably because at the time, cyclosporin was not in use for long enough to make any definite conclusions. The use of azathioprine is thought to be responsible for an increased risk of skin cancer due to the presence of its active metabolite 6-Thioguanine nucleotide in erythrocytes, supporting chemical carcinogenesis as a likely cause (46).

Other forms of immunosuppressive therapy used are the monoclonal antilymphocytic agents, OKT3, ALG and ATG. These are used mainly as induction therapy and for episodes of refractory rejection. While there is little data on their possible cutaneous oncogenic potential, the use of OKT3 has been minimised in many centres because it has been shown to result in a high incidence of lymphoproliferative cancers (65). Further studies are required to define the risk of skin malignancies of this subgroup of very strongly immunosuppressed patients.

Ultraviolet light exposure

Sunlight is an important risk factor in the development of skin cancer and keratoses both in the immunosuppressed transplant patients and the nonimmunosuppressed population (13, 47) whereas it is a risk factor for the occurrence of warts only in the immunosuppressed transplant population (13, 87). Viral warts in transplant patients are also significantly associated with pale skin which burns on ultraviolet light exposure (53). High exposure to sunlight before the age of 30 is more significant as a risk factor for the development of skin cancer by 45 years, than exposure after the age of 30 (13). In contrast, there appears to be no association between life-time exposure to sunlight and the number of keratotic skin lesions (13). A recent study by Gallagher *et al* in the immunocompetent patient population suggests that recent sun exposure is more important than cumulative lifetime exposure in the development of skin cancers (32). The speed of development of skin cancers is related to the latitude of each transplant centre, skin cancers appearing earlier with decreasing latitude (20, 79). Ultraviolet light exerts its effects by damaging DNA leading to gene mutations in cutaneous cells which results in

malignant transformation. Since U.V light induces cutaneous immunosuppression through various mechanisms such as affecting the function of antigen-presenting cells, inducing the release of cytokines and modulating the expression of surface molecules (88), the host is unable to recognise and destroy newly transformed cells. It also induces systemic immunosuppression by generating cis-urocanic acid close to the skin (58). This cumulative effect of U.V light induced coupled with drug-induced immunosuppression encountered in the transplant recipient could greatly amplify the risk of development of skin cancer. There are no studies so far that have defined this cumulative risk or have categorised people into high risk or low risk groups depending on their skin type, degree of sun exposure, family history and personal history. This needs to be studied in detail since it could be useful in instituting preventative measures in the high risk group.

Human Papillomavirus infections

Cutaneous lesions: Human Papillomavirus (HPV) infections like warts are the commonest post transplant complications. The HPV types implicated most commonly are HPV 2 and 4, occasionally other types. SCCs may follow warts after an interval of two to three years causing speculation that warts in immunosuppressed patients could predispose to malignancy, forming a continuum of disorders (13, 57, 83). This is however far from proven and is mere speculation. HPV has been implicated in the development of skin cancers in these individuals as it is found frequently in dysplastic and malignant squamous cell lesions (6, 27, 80). Earlier European studies (6, 26, 27, 29, 69, 85) reported HPV types 5 and 8 as being mainly responsible for skin cancer particularly SCCs. Australian studies however were not been able to substantiate this correlation (8, 23). It is not clear whether

this discrepancy was geographical in origin . Five years later one of the European groups (6) found HPV types other than 5 and 8 in renal allograft recipients (77). This is possibly because of the development of more sensitive and specific present day techniques for HPV detection and analysis. HPV types that have been isolated by further refinement of laboratory techniques are 12, 20, 23, 38 and 2 novel types related to HPV 10 and 38 (21, 40). The fact that HPV types have been detected in some skin cancers and not others might mean that unidentified types could be present in HPV 'negative' lesions which require detection by further refinement of laboratory techniques or that they might be present only in low copy number and hence below the level of detection. McGregor *et al.* however believe that p53 gene mutations are the major determinants of posttransplant skin malignancies as opposed to HPV induced p53 degradation and the presence of HPV in skin cancers is coincidental (51). p53 is believed to accumulate in skin cancers, specifically in areas of epidermal dysplasia but immunocytochemical staining has shown that there are factors other than p53 gene mutations which cause accumulation of p53 in skin cancers (78).

Important evidence of an association between HPV and both cancers and precancers comes from the histopathological examination of squamoproliferative lesions. The majority of them show cellular features of viral infection together with bowenoid changes and abnormal multinucleate cell (6, 67). This is in contrast to classical SCCs in the non immunosuppressed population. A recent study from the U.K however suggests that it is not possible to distinguish between squamoproliferative lesions in the immunosuppressed or immunocompetent on a histopathological basis (33). Strong evidence for the association of the cutaneous oncogenic potential of HPV is also

evident from the occurrence of HPV types 5, 8, 9, 14, 17 and 20 in the SCCs of patients with the rare inherited condition Epidermodysplasia Verruciformis (11, 39, 71). While there is no data on HPV association with skin cancer in heart and heart-lung recipients, the data on even more extensively studied kidney graft recipients remains inconclusive and the strength of the possible association requires additional study.

Anogenital lesions: Previous studies have shown a 14 to 16 fold increase in the incidence of cervical cancer (2, 63) and a 100 fold increase in the incidence of vulval and anal cancers (63) in renal transplant patients compared with the normal population. Genital cancers tend to occur later in the course of immunosuppressive therapies as compared to other malignancies. 'Low risk' HPV types 6 and 11 and oncogenic HPV types 16 and 18 have been implicated in the occurrence of cervical warts and all grades of dysplasia (2, 55). HPV types 16/18 have been identified in anogenital and cervical carcinomas in most studies, thereby corroborating the functional role of human papillomaviruses in their development (84, 87). HPV is not only associated with cervical cancer but plays a definite aetiological role in the development of the disease. This role is further strengthened by the consistent detection of koilocytosis, a histological feature of viral infection, in genital lesions of immunosuppressed patients (13, 36). HPV 16 and 18 have transforming gene products such as E6 and E7 which interact with tumour suppressor proteins like p53 and PRB respectively and promote carcinogenesis. The mechanism of carcinogenesis if any, in skin cancers is likely to be different from HPV types 16 and 18.

A number of questions remain unanswered with regard to what extent the above risk factors contribute to the causation of skin cancers. There are suggestions from a few studies that the HPVs found in skin cancer have a low transforming potential and therefore are incapable of acting as carcinogens on their own. McGregor *et al.* hypothesise that viral infection cannot be the only causative factor in BCCs and SCCs by virtue of its presence alone, because it does not explain the predominance of SCCs over BCCs (52), although according to another school of thought only viral infection could explain the increased incidence of squamous cell carcinoma over basal cell carcinoma in transplant patients (47). The role of sunlight as a co-carcinogen in the development of skin cancers in transplant and non transplant patients is very well established by all groups.

Other risk factors

Age - It has been shown in renal allograft recipients that the risk of SCCs is greater in those transplanted after the age of 35 years (22, 23).

HLA type - A Dutch study (17) showed increased risk of SCC with HLA-B mismatching and HLA-DR homozygosity in renal allograft recipients. Furthermore, positive associations with skin cancer has been found in these patients with HLA-B27 and HLA-DR7 (20) and a negative association with HLA-A11 (15). HLA-B mismatch and HLA-A11 seem to have no effect on the occurrence of cutaneous warts (10). A recent Australian study (61) has shown that HLA-DR homozygosity was associated with skin cancer overall, HLA-DR7 was protective factor in skin cancer and HLA A1 and A11 were protective factors in Bowen's disease. Two other studies (89, 90) failed to demonstrate a positive association with HLA-DR1 and A3 or a

negative association with HLA-A11 in renal transplant patients with skin cancer.

Sex: Male recipients of kidney grafts are thought to have a higher incidence of skin cancer than females (47) but this conclusion was drawn without correction for UV exposure differences.

Ethnic variations: Higher prevalence of cutaneous cancers has been found in transplant recipients in South Wales as compared to rest of the U.K. probably owing to their higher proportion of Celtic ancestry (44).

Immunoglobulin levels: This has not been widely studied. Pollock *et al* (66) have shown higher incidence of skin malignancies in renal transplant recipients with low immunoglobulin levels.

c) Prevention and treatment options

Prevention : Prevention of cutaneous problems post-transplant is imperfect at this time. Patients should be educated about the importance of avoidance of UV exposure and the use of sun protection, but substantial damage to the skin may have been done in the years previously. However, findings of Gallagher *et al* (32) suggest that recent sun exposure may be more relevant than cumulative lifetime exposure, making it of paramount importance that this group of patients, should be strongly advised about sun protection and avoidance.

Treatment options: Oral retinoids have been used to reduce the risk of development of skin cancers in renal transplant recipients. The exact mechanism of action of the oral retinoids is unclear and different mechanisms have been suggested by various studies. One mechanism suggested is immunomodulation via stimulation of T-cell

function which is an important component of their anti-tumour effect. Retinoids are also thought to have a direct effect on glycoprotein synthesis in epidermal cells and can induce tumour cell necrosis. Etretnate is also thought to act selectively on abnormal sun-exposed epidermis. Both controlled and uncontrolled studies of oral retinoids in renal transplant recipients suggest that they may cause regression of warts, keratoses and skin cancers without any significant adverse effects. Most patients do however experience side effects such as chelitis, generalised dryness and muscle aches (16, 76, 85). A controlled study has shown that oral retinoids in a dose of 30mg/day over 6 months can significantly reduce the incidence of keratotic skin lesions and prevent the occurrence of squamous cell carcinoma (16). Another recent study has also demonstrated the benefits of topical tretinoin, which with long-term use over a period 9 months, can prevent the occurrence of new lesions and reduce any existing ones (68). The use of retinoids may however be restricted in the heart transplant population who are at a greatly increased risk of accelerated atherosclerosis, as they result in elevation of serum lipids.

Surgical removal of skin tumours is sufficient in most patients, but therapeutic problems arise when lesions tend to be multiple and rapidly recurring. Extensive excision of severely dysplastic skin and grafting with non sun-exposed skin may be warranted in renal transplant recipients with SCCs, due to their aggressive nature.

The future

The future looks bright and a lot of work is presently being done to induce states of immune unresponsiveness directed towards foreign antigens of the allograft, thereby doing away with long term immunosuppressive therapy and making post-transplant malignancies

a thing of the past. Future research will probably also look into interactions between viral and host-cellular proteins (52).

1.1.2 Human Papillomaviruses

Human papillomaviruses are small, non-enveloped, icosahedral, viruses that replicate in the nucleus of small epithelial cells. Papillomavirus particles are 52 to 55nm in diameter and contain circular, double stranded DNA of about 8000 base pairs (Fig1). Even after their morphology came to light with the advent of the electron microscope in the 1960s, their characterisation took a while because they cannot be grown in tissue culture.

To date over 80 different of papillomaviruses have been isolated and characterised with the help of molecular biology techniques. Specific clinical entities caused by them have been defined. Papillomaviruses are species specific and have cellular tropism for squamous epithelial tissue. Susceptible cells respond to viral infection by causing cellular proliferation which is the fundamental nature of papillomaviruses (Fig 2).

The genome organisation of the majority of the HPV types consists of at least 7 early (E) genes (E1 to E7) and two late (L) genes (L1 and L2). E genes are expressed early during the process of infection of an epithelial cell and the L open reading frames (ORFs) are expressed only in productively infected cells (Fig 1).

E1 and E2 are viral regulatory proteins, involved in viral DNA replication and viral transcription.

E4 proteins complexes with cytokeratins and causes them to collapse. E5, E6 and E7 proteins of the papillomavirus have transforming activities through binding with p53 and pRB.

L1 is the major capsid protein and L2 is the minor capsid protein. L1 is 500 amino acids in size, is highly conserved among the various papillomavirus types and is thought to mediate the major humoral and cell mediated responses to infection.

Histologically the lesions caused by human papillomavirus manifest in the following ways:

1. Thickening of the epidermis (acanthosis).
2. Hyperkeratosis.
3. Some amount of papillomatosis.
4. Presence of keratohyalin granules in the granular layer of keratinised epithelium.

Fig 1

Genomic map of HPV type 16

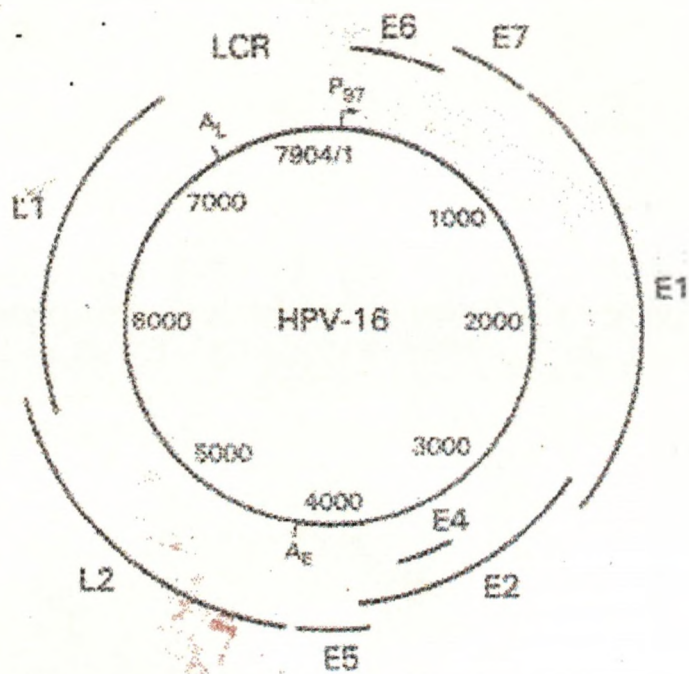
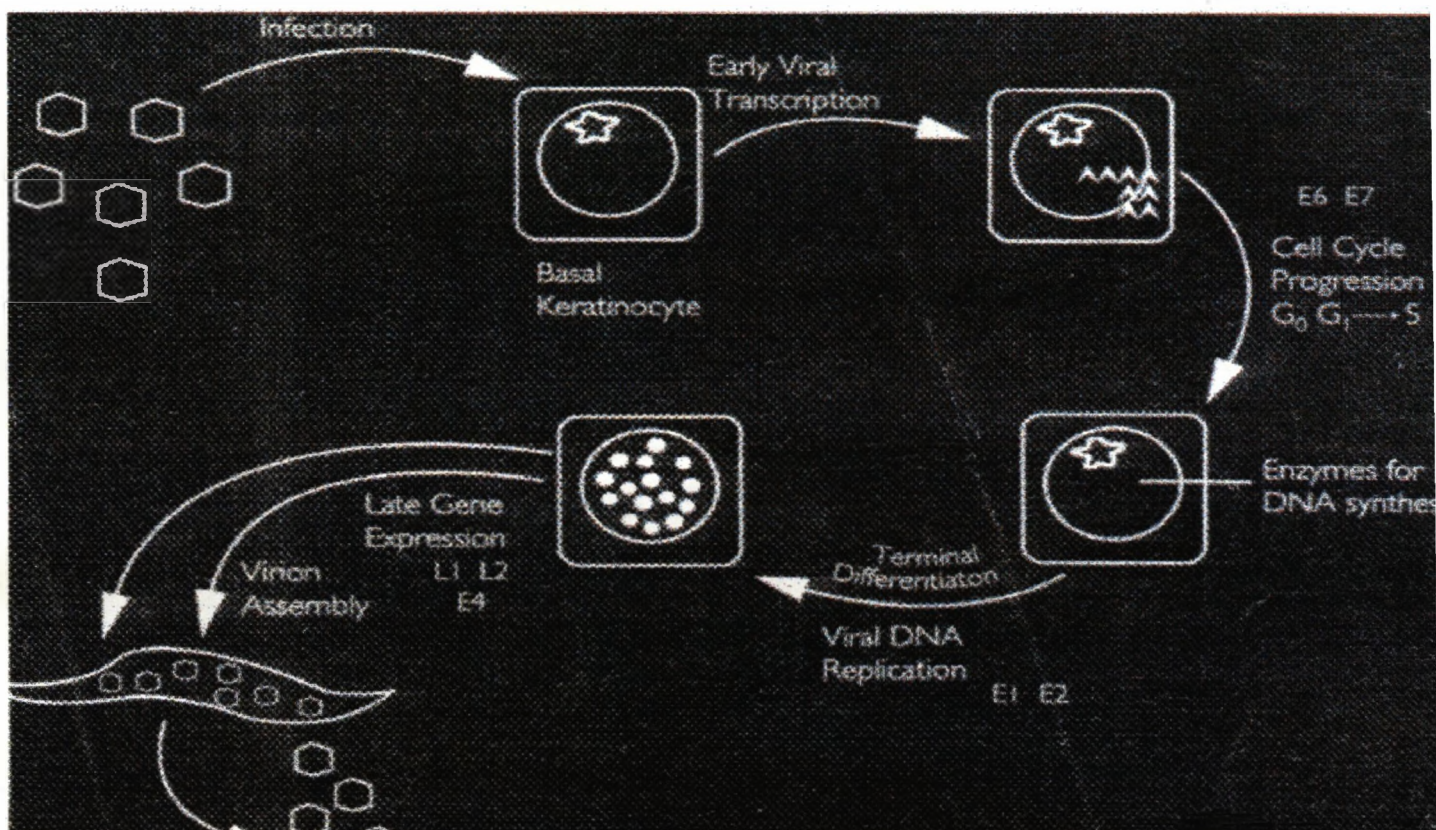


Fig 2

Course of an HPV infection resulting in wart formation



1.2 Aims of the study

The aims of this study are fourfold:

1. To define the risk of malignant and premalignant skin lesions in heart, heart-lung and lung transplant recipients and to determine the prevalence of warts and keratoses in this group of patients.
2. To evaluate the presence of human papillomavirus types in these lesions in such patients.
3. To correlate the level of immunosuppression and immunological events with the appearance of HPV and subsequent skin cancers.
4. To identify other significant risk factors in the development of skin cancers in these patients.

A total of 740 heart transplants, 230 heart-lung transplants, 102 single lung transplants and 31 double lung transplants have so far been carried out at Papworth Hospital in the last 15 years. All surviving patients and medical records of those who died one or more years after transplantation were assessed with respect to skin disease. The clinical data analysis consisted of two major parts:

Retrospective analysis of patient data

Data collection was been done by means of

- a. Patient Questionnaire : This was completed by the patient and formed the basis of the initial assessment. The information obtained helped in identifying patients with specific skin problems, in particular problems such as viral warts, keratoses and skin cancers and gave some indication of potential risk factors including exposure to

ultraviolet radiation and family history. Questionnaires were collected from all surviving transplant recipients.

b. Patient records : Detailed data was collected from patient records held at Papworth hospital and supplemented with information obtained on request from the patients' physicians. Data collection was done to assess the role of various risk factors in causing the skin lesion.

c. Interview and examination : Patients were seen individually and further details documented of life-time sun exposure, pre-transplant skin disease, development of skin cancers, warts and pre-cancerous lesions post-transplant. Examination of patients' skin helped in both qualitative and quantitative assessment of skin disorders, at least at one time point for each patient.

Determination of the role of HPV

a. Analysis of diseased tissue: Scrapings or biopsied specimens from all existing and new cutaneous lesions such as warts, keratoses and skin cancers were processed for the type and presence of HPV.

Most studies so far have focused on long term skin complications in kidney transplant patients. There are few studies on the spectrum and incidence of skin problems encountered in heart transplants and practically none in the heart-lung and lung transplant group. They differ from the kidney group in the following ways.

a. Heart transplant recipients are very often more ill pretransplant

b. Immunosuppression tends to be higher in heart transplant patients than kidney transplant patients and even higher in the heart-lung group.

c. Heart-lung transplant patients are younger than the heart and kidney transplants.

Since there have been no conclusive results from various studies on the presence of human papillomavirus in skin cancers especially SCCs, it was a useful outcome of the study to document this in the heart and heart-lung transplant group.

Chapter 2

MATERIALS AND METHODS

2.1 Laboratory methods

General Methods

2.1.1 DNA extraction

- a. DNA extraction from paraffin embedded tissue
- b. DNA extraction from frozen tissue
- c. DNA extraction from wart parings and frozen wart sample from patients in routine dermatology clinics.

2.1.2 Polymerase Chain Reaction

2.1.3 Agarose Gel Electrophoresis

2.1.4 DNA purification and Gel Extraction

2.1.5 Cloning with a TA Cloning Kit

- a. Addition of overhangs post amplification
- b. Ligation reaction
- c. Transformation

2.1.6 Mini-prep methods

- a. Mini-Prep methods for the overnight cultures

- b. Eco RI check digestion

2.1.7 DNA Sequencing

2.1.8 Sequence Analysis

2.1.9 Materials

2.2 Clinical methods

2.2.1 Retrospective Analysis of patient data

- a. Patient Questionnaire

- b. Patient records

- c. Interview and Examination

2.2.2 Determination of the role of HPV

- a. Analysis of Diseased tissue

Materials and Methods

2.1 Laboratory Methods

General methods

Laboratory methods were used for the purpose of detecting human papillomavirus DNA within the skin tumours of thoracic transplant recipients. DNA was extracted from tissue for amplification by polymerase chain reaction. Tissue was available in different conditions: a) formalin-fixed, paraffin-embedded biopsies, b) fresh, frozen biopsies and c) fresh wart parings. Different extraction methods were applied, as detailed in sections 2.1.1.a, 2.1.1.b and 2.1.1.c respectively.

DNA extraction was followed by the polymerase chain reaction in which target DNA sequences are amplified through repeated cycles of denaturation, annealing with primers complementary to flanking regions of the target sequence and finally DNA synthesis. This produces enough DNA to be tested adequately because it amplifies a single DNA molecule into billions of molecules. Each temperature cycle requires between 1-5 minutes. Thus 20-50 cycles are needed to produce the desired molecular sensitivity take 2-4 hours. A typical PCR amplification is capable of producing 10^{12} molecules of HPV amplified DNA per 100µl of reaction.

The development of the polymerase chain reaction, *in vitro* gene amplification technology, is a major virological advance in molecular

biology. The polymerase chain reaction is considered the method of choice for detection of human papillomavirus in clinical samples because of its sensitivity and significantly reduced assay time. Amplifying the fragment required and visualising it on agarose gel is possible in a matter of just a few hours.

Techniques such as Southern blot, filter and in situ hybridization have been used in the past with great success but they have their limitations because of the large amount of DNA material required for HPV detection and therefore their use is limited in archival specimens. The maximum overall prevalence of HPVs has been seen with PCR. The polymerase chain reaction thus seemed the most suitable technique for the study.

2.1.1 DNA Extraction from tissue

2.1.1.a DNA Extraction from paraffin tissue

The paraffin blocks containing embedded fixed biopsy tissue together with the clinical and histopathological report were obtained from the patient's dermatologist or via the histopathology department.

25µm sections were cut on the micrometer and 2 sections were stored in a 1.5 ml eppendorf tube. To avoid contamination, a plain wax block was cut in between different tissue blocks, and a separate blade was used for each block and the cutting surface cleaned regularly with xylene. The sections were stored at room temperature.

Protocol a)

1. Paraffin was removed from sections by the addition of 300µl of xylene. After 2 minutes, the mixture was centrifuged briefly and the xylene removed.
2. 300µl ethanol was added, to remove the remaining xylene, and then discarded. Residual ethanol was evaporated from the tissue sections by incubation at 55°C with an open lid.
3. 50µl of DNA extraction buffer (50mM Tris HCl pH 8.5, 1M EDTA, 0.5% Tween 20) plus 1.3µl of proteinase K stock solution (20mg/ml) were added to digest the DNA and incubated at 55°C overnight.
4. The proteinase K was inactivated by incubation at 95°C for 10 minutes.

5. The samples were rapidly cooled on ice and stored at -20°C until use. 5µl of this digested mixture was used in a PCR reaction.

Solution used:

x 20 Extraction Buffer

50mM Tris HCl, pH 8.5

1M EDTA

0.5% Tween 20

pH adjusted to 8.5 with HCl

Protocol b)

1. Sections were deparaffinated as described in steps 1-2 of Protocol a)
2. PCR reagents were added directly to the dewaxed tissue. (Anticipated success rate for optimal 5 square mm of section is 70%)

Protocol c)

The tissue was treated as in steps 1-5 of Protocol a) and then a phenol / chloroform / ethanol extraction was performed as described in part 2.1.1.b below.

All 3 methods were tried in order to determine the most suitable method which gave the best results. Protocol a) gave the best results and was finally used for all the samples.

2.1.1.b DNA Extraction from frozen tissue

1. Fragments of frozen tissue were homogenised in 1ml of 'Frozen Tissue Buffer' and rapidly refrozen.
2. 100µl of 10% SDS and 20µl of proteinase K (10mg/ml) were added and the tissue digested at 50°C for 5-16 hours.

3. The resulting digest was divided equally into two, and each mixed with an equal volume of phenol. The aqueous phase, containing DNA, was removed and the remaining phenol dissolved with addition of chloroform.
4. The upper aqueous phase was again removed and mixed with three times the volume of ethanol and one tenth the volume of 3M Sodium acetate, pH 4.8. The solution was frozen at -20° C overnight or -70°C for half an hour and then spun for half an hour and the pellet visualised.
5. The ethanol was removed, the pellet rinsed in 70% ethanol, vacuum dried and resuspended in 30µl TE buffer or water after which the was subjected to PCR amplification.

Solutions used:

Frozen Tissue Buffer

0.5 M Tris HCl

0.1 M Na acetate

0.2 M EDTA

The final pH of the solution was 8.0.

10% Stock solution of sodium sarcosine

10 g of N-lauryl sarcosine was added to a final volume of 100 ml of water and dissolved overnight to make a 10% solution.

2.1.1.c DNA extraction from wart parings and frozen wart samples from patients in routine dermatology clinics.

The efficacy, sensitivity and specificity of the primers and the PCR reaction was assessed from wart specimens obtained from routine dermatology clinics.

Wart parings were washed in 1 ml cold lysis buffer and resuspended in 450µl of cold lysis buffer and 50µl of lysis solution which contains Proteinase K for DNA extraction. The DNA solution from the frozen samples was also treated with a phenol / chloroform / ethanol extraction as described in 2.1.1.b.

Solutions used:

Lysis Buffer

10mM Tris HCl, pH 7.5

10mM EDTA

Lysis solution

50µl lysis buffer

12.5µl of 20mg/ml of Proteinase K

30µl of 10% SDS

TE Buffer (10:1)

10ml 1M Tris-HCl, pH 8.0

2ml 0.5M EDTA

Distilled water to 1000ml

0.5 M EDTA, pH 8.0

Phenol TE-saturated phenol (Sigma) was aliquoted and stored at -20°C.

2.1.2 Polymerase Chain Reaction

The PCR reaction mixture consisted of the following:

Constituents	50ul reaction mixture	20ul reaction mixture
1. x 10 PCR Buffer	5µl	2µl
2. 25mM MgCl ₂	5µl	2µl
3. 2mM dNTP	7µl	2.5µl
4.Oligonucleotide primer 1	2µl	2µl
5. Primer set 2	2µl	2µl
6. Taq Polymerase enzyme	1µl	1µl
7. DNA solution	5µl	2µl
8. Water	23µl	6.5µl

The components were mixed gently before placing in the thermocycler. Temperature cycling was done according to the following steps of 30 cycles each. The number of cycles set are determined by the amount of template DNA in the reaction, with less template DNA the number of cycles need to be increased.

1. Initial denaturation, which is of paramount importance, was done for 1 minute at 95°C. This is so because a complete denaturation results in more efficient utilisation of the template and therefore a better yield of the PCR product. Taq Polymerase was usually added before the start of the PCR since it was only at 95°C for 1 minute. If the Taq polymerase is subjected to that temperature for longer periods it loses its stability, despite its relative thermostable nature. Taq DNA polymerase has a propensity for adding non-templated

nucleotides to the 3' end of DNA chains. This can result in problems during cloning the PCR product.

2. Annealing was done for 1 minute at 56°C. This is usually determined by the GC content of the primers used. If the GC content is less than 50%, annealing is done at 55%, if the GC content is more than 50%, annealing is done at 60°C. This step ensures that the primers anneal to the template stably.

3. Extension was set at 72°C for 1 minute. This is determined by the expected product length. If the expected product length is ≤ 500 , extension is one minute. For products >500 nucleotides, extension is 3 minutes.

4. The extension time was further increased to 7 minutes at 72°C to ensure the full length of the PCR products.

5. The holding capacity of the PCR thermocycler is 99 hours and the samples could be kept for that length of time at 4°C but in practice was no more than 16 hours.

The conditions of the polymerase chain reaction had been used in the laboratory for amplification of HPV DNA. For the purposes of amplifying DNA from tissue, the conditions were not changed as they produced a detectable PCR product.

Solution used

2mM dNTP

2mM solution of dNTP was made from 10mM of dATP, dCTP, dGTP and dTTP. 100mM stock solution of the above was diluted to 10mM.

2.1.3 Agarose Gel Electrophoresis

A 1% multipurpose agarose horizontal mini-gel was prepared and submerged in TBE buffer. DNA samples with 1/6 volume of the DNA loading buffer were loaded into the wells. A current of 70mA was passed across the gel for half an hour.

Method

1. 10ml of TBE Buffer was diluted with 90 ml water.
2. 0.50 gms of the multipurpose agarose was added to 50ml of the diluted buffer solution. Ethidium bromide 1-2 μ l at a concentration of 25mg/ml was added to the remaining buffer.
3. The agarose mixture was heated, cooled and added to the gel tank to set and then the diluted buffer solution added over the set gel before application of current.

Solutions used

TBE Buffer x10

89mM Tris base

89mM boric acid

2.6mM EDTA

pH adjusted to 8.3 with HCl

DNA loading buffer

0.25% Bromophenol Blue (runs at 400 bp)

50mM EDTA

30% Glycerol

0.04 M Tris HCl, pH 7.0

0.02M sodium acetate

1 kb ladder Stock solution (1ug/ul) diluted 1:20 in DNA loading buffer.

2.1.4 DNA purification and Gel Extraction

This is done for the efficient purification of PCR products from amplification reactions and to contribute as a first step in DNA purification towards the final step of DNA sequencing.

Method of gel extraction

1. 42µl of a 50µl PCR reaction mixture was mixed with 5µl of loading dye and subjected to electrophoresis in a 1% agarose mini-gel.
2. The DNA band was visualised under low wavelength UV light and the gel cut just in front of the DNA band in order to insert a small strip of treated DEAE paper.
3. The gel was run for a further 15-20 minutes and confirmation that the DNA had transferred on to the paper was obtained by visualisation under UV light.
4. The DEAE strip was rinsed in NTE1 solution to remove agarose and then placed in 500µl NTE 2 solution and incubated at 50° C for 30 minutes. This incubation was performed so that the DNA would be eluted from the DEAE paper and into solution.
5. The paper was transferred to a fresh eppendorf tube, and given a final wash with 50µl of NTE 2 which was added to the 500µl NTE 2 buffer.
6. Of this 550µl, 225µl was subjected to a phenol/chloroform/ethanol extraction. The DNA was precipitated with 2.5 times the volume of ethanol (1250µl) and 3µl 3M NaAc.
7. The DNA was kept at -70°C for half an hour, centrifuged for 10 minutes and the pellet resuspended in 30µl of water.

Reagents used

Treated DEAE paper

DEAE paper was cut into strips and washed for 10 minutes in 10mM EDTA (pH 7.6), 5 minutes in 0.5 M NaOH and then in distilled water several times. The prepared strips were stored in water.

NTE 1

0.15M NaCl

20mM Tris

0.1mM EDTA

NTE 2

1M NaCl

20mM Tris

0.1mM EDTA

2.1.5 Cloning with a TA Cloning Kit

The TA Cloning Kit (Invitrogen) was used to clone the products of PCR amplification. The DNA obtained by PCR from tissue was ligated after gel extraction into a pCR vector and then transferred into competent *E.coli* cells.

2.1.5.a Addition of overhangs post amplification

Usually if Taq polymerase is used for the PCR reaction the cloning efficiency is good and the 3' A-overhangs are not removed. If however the blunt-ended fragments need to be cloned, the following need to be added:

1. Add 1µl of Taq polymerase per tube was added and incubated on heat block equilibrated to 72°C for 10 minutes.
2. The DNA was extracted with equal volume of phenol/chloroform, 1/10 volume 3M Sodium acetate and twice the volume of absolute ethanol then added. The mixture was centrifuged, the DNA pellet air dried and resuspended in 40µl of water. The addition of 3'A overhangs was done only in a few samples. The procedure did not add to the cloning efficiency and so was not adopted as a routine.

2.1.5.b Ligation reaction

A 10µl ligation reaction mixture was prepared

- | | | |
|----|---------------------|-----|
| 1. | Fresh PCR product | 2µl |
| 2. | x10 Ligation Buffer | 1µl |
| 3. | pCR 2.1 vector | 2µl |
| 4. | Sterile water | 4µl |

5.	T4 DNA Ligase	1µl
	Total volume	10µl

Ligation reactions were incubated at 14° C overnight.

Precautions taken

1. No more than 2-3µl of the PCR in the ligation reaction was used so that T4 DNA ligase was not inhibited by salts in the PCR
2. The ligation reaction was kept at 14°C to maximise the accuracy of the ligation efficiency.

2.1.5.c. Transformation

Two types of cells are provided with the TA Cloning kit - INV ALPHA F'
- TOP 10 F'

Before starting with the Transformation reaction the following steps were taken:

1. A water bath was heated to 42°C, a vial of SOC was thawed and brought to room temperature.
3. The plates made of a mixture of 2TY+ ampicillin in agar were taken out from the cold room and kept at 37° C and then brought to room temperature.
4. 40µl of 40mg/ml of X-Gal was added to each plate and then spread evenly.
5. Whenever TOP10F' competent cells were used, 40µl of a 100mM IPTG solution was added to the plate along with X-Gal. This was to enable blue-white screening of bacteria containing inserts in the vector. The liquid was properly soaked on to the plates.

Method

1. The ligation reactions were centrifuged for a few seconds and kept on ice.
2. A vial of 0.5M β -mercaptoethanol and competent cells were thawed for each transformation.
3. 2 μ l of 0.5M β -mercaptoethanol was added to the vial of competent cells and mixed by stirring gently with the pipette tip.
4. 2 μ l of ligation reaction was added into the cells and mixed gently.
5. Vials were kept on ice for 30 minutes, heat shock given for 30 seconds in a 42°C water bath, and then put back on ice for 2 minutes.
6. 250 μ l of the SOC medium (provided with the kit) was added at room temperature to each tube and the vials were kept in a 225rpm shaker at 37° C for 1 hour.
7. 50 μ l and 200 μ l of each transformation mix was spread on to separate labelled plates. Plates were inverted and left for an hour before overnight incubation at 37°C.

Analysis of the Transformants

1. Each transformation reaction was analysed for the presence and number of white colonies and overnight cultures were set up for plasmid isolation.
2. White colonies were picked from sites closest to blue colonies.
3. Colonies were grown in 2-5 ml of 2TY broth to which 50 μ g/ml of ampicillin was added. Incubation was done either by means of a wire loop or sterile pipette tips and kept overnight in a shaker for good agitation.

Difficulties encountered and Precautions taken

1. White colonies did not always have an insert. A repeat transformation with another tube of the vector was performed.
2. Only white colonies without any blue colonies were obtained. IPTG may have been inadvertently omitted.
3. White colonies of normal size were surrounded by smaller white colonies. These are satellite colonies and were not picked as they did not have insert.
4. Sometimes very few white colonies were obtained and majority of the colonies were blue or light blue.

This could be due to a number of reasons:-

- The PCR products were kept for a long time before the ligation reaction and that could have reduced the efficiency.
- Too much of the amplification reaction was added to the ligation; not more than 2-3µl needed to be used.

Solutions used

Stock solution of Ampicillin (5mg/ml water). Stored in aliquots at - 20°C.

Stock solution of X-gal at 40mg/ml in dimethylformamide. Kept at -20°C. Protected from light.

2xTY Medium

16g Tryptone

10g Yeast extract

5g NaCl. In 1 litre water. Autoclaved and stored at room temperature.

2.1.6 Mini-prep methods

2.1.6.a Mini-Prep methods for the Overnight Cultures

Two different methods were used for the preparation from bacteria and screening of plasmid DNA. The DNA produced by Method 1 was of inadequate purity for sequencing and so a second method was adopted.

Method 1

1. Bacteria in 3ml of broth cultured overnight were pelleted by centrifugation for 1 minute and the supernatant discarded.
2. 100µl of lysis solution was added to the pellet and the bacteria resuspended.
3. 200µl of alkaline-SDS solution was added, mixed and left on ice for 2 minutes.
4. 150µl of high salt solution (3M sodium acetate, pH 4.8) was added, mixed thoroughly and left on ice for 45 minutes. The mixture was vortexed every 15 minutes.
5. After centrifugation in a microfuge for 5 minutes, the supernatant was transferred to a fresh eppendorf tube and 1ml of cold ethanol was added, mixed thoroughly and left on dry ice for 10 minutes.
6. The mixture was centrifuged 5 minutes and the supernatant discarded. The DNA pellet was rinsed with 95% ethanol, dried and redissolved in 0.2ml 0.1M sodium acetate, pH 7.0. The DNA was reprecipitated with 0.5ml cold ethanol at -70°C for half an hour or -20°C overnight.

7. Finally the solution was centrifuged for 15 minutes and the pellet rinsed with 70% ethanol, dried in a dessicator and dissolved in 30-40µl sterile H₂O.

Solutions used

Alkaline SDS solution

20ml of 1M NaOH

10ml of 10% SDS

Distilled water to 100ml

Method 2

The other method used was a Wizard Mini-prep kit. This gave DNA of pure quality which could be sent off for sequencing directly.

1. Cells were pelleted from 2ml of overnight culture and resuspended in 200µl of cell resuspension solution. 400µl of Neutralisation solution was added and mixture centrifuged for 5 minutes to obtain a clear lysate.

2. The cleared lysate was transferred to a fresh tube to which 1ml of Wizard miniprep resin was added and mixed.

3. The mixture was transferred to a Wizard mini column and liquid removed by vacuum.

4. The resin was washed with 2ml of 40% isopropanol/4.2M guanidine hydrochloride, especially for end A strains and then with 2ml of column wash solution.

5. 40µl of water was added and after 1 minute, the eluted DNA was collected by microcentrifugation for 20 seconds.

2.1.6.b Eco RI Check Digestion

A restriction enzyme digestion was performed and the DNA visualised under UV light after electrophoresis in an agarose gel. This was done as a check to visualise and therefore to confirm the presence of DNA in the mini-prep. sample before sending it off for sequencing.

Mini-prep DNA sample	15µl
Eco R1 enzyme (20,000 iu/ml)	1µl
x10 digestion buffer	2µl
RNase	1µl
H2O	1µl
Incubation:	37° C for an hour.

Measurement of DNA

The amount of DNA present in the sample was determined spectrophotometrically before subjecting to sequencing at a concentration of 10ng/ml.

2.1.7 DNA Sequencing

Sequence data was obtained, with the help of M13 forward and reverse universal primers. The DNA was sequenced using 2 different automated sequencers. Both of them were supplied by Perkin-Elmer Applied Biosystems and sequencing was performed in the Biochemistry laboratories.

1. ABI Applied Biosystems Prism 373 XL -15 hour run.
2. ABI Applied Biosystems Prism 377 XL - 8 hour run.

2.1.8 Sequence Analysis

A search was performed to find a homology between the sequences obtained from positive DNA samples and those present in the EMBL database. The programme used for the search was Fasta and that was provided in the software by Wisconsin Package version 9.1, Genetics Computer Group (GCG), Madison, Wisconsin.

Another programme from the same package called Gap was utilised which uses algorithm of Needleman and Wunsch (48) to find the alignment of two complete sequences that maximises the number of matches and minimizes the number of gaps.

2.1.9 Materials

General purpose reagents were supplied by Sigma, Boehringer Mannheim, East Anglia Chemicals, BDH Chemicals, Fisons, Pharmacia, AnalaR and Bethesda Research laboratories (BRL).

Enzymes

- | | |
|------------------------|--------------------------------|
| 1. Taq DNA Polymerase | Boehringer Mannheim |
| 2. Restriction Enzymes | Boehringer Mannheim
Biolabs |

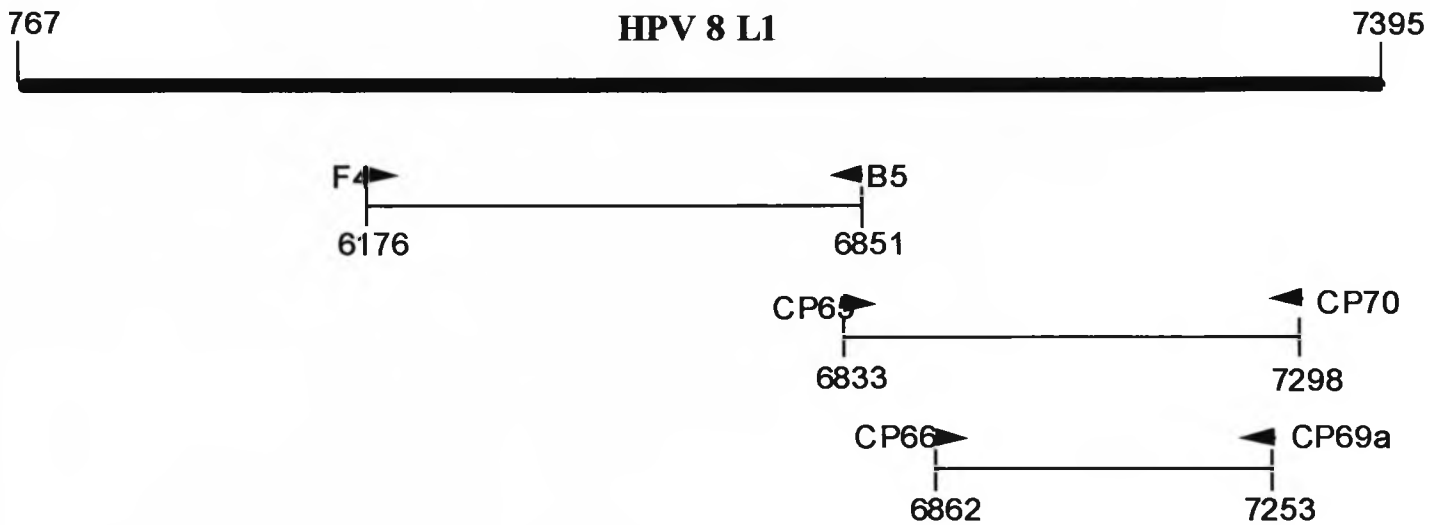
Gibco BRL Custom primers (Nested primer pair - CP) were obtained from Life Technologies, Ltd. Primers and were designed according to the Berkhout nested primers. (10).

Degenerate Shaminin (Sha) primers were obtained from Perkin-Elmer Applied Biosystems UK and were designed according to the German group to enable detection of a broad range of papillomavirus types (9).

Both the above were designed from the L1 region of the Open Reading Frame since that is the most conserved region among the various HPV types.

Primers were supplied lyophilised and resuspended at a concentration of 10 pmol/μl (forward primer) and 10 pmol/μl (reverse primers) prior to use.

Alignment of sequences of Human papillomavirus 8 with
homology to the oligonucleotide Berkhout primers.



CP 65
6832-
6851:CAGGGTCATAATAATGGCAT.....GATCAATATTCATTAGGCAGG

Table 1. Oligonucleotides used for the polymerase chain reaction

Primer name	Sequence 5'-3'	Primer Length
CP 65	CAR GGT CAY AAY AAT GGT GGY AT	20
CP70	AAY TTT CGT CCY ARA GRA WAT TGR TC	20
CP 66	AAT CAR MTG TTT RTT ACW GT	20
CP 69A	TCW GTY ATR TCT ACA TYC CA	20
CP 62	GTW AAT GAA AYT TGY AAA TAT CC	23
Sha B5	CTG GAT CCA YNC CRT TRT TRT GNC CYT G	28
Sha B6	CTG GAT CCA YNC CRT TRT TYT GNC CYT G	28
Sha B11	CTG GAT CCA YNC CRT TRT TYG TNC CYTG	28
Sha FP	CTG GAT CCT CNM GNG GNC ANC ANC CNY TNG G	28
beta globin F	GAA GAG CCA AGG ACA GGT AC	20
Beta globin R	CCA CTT GCA CCT ACT TCA AC	20

- M= AC
- R= AG
- W=AT
- S= GC
- Y= CT
- K= GT
- V= ACG
- H= ACT
- N= ACGT

2.2 Clinical Methods

Plan of work: A total of 740 heart transplants, 230 heart-lung transplants, 102 single lung transplants and 31 double lung transplants have so far been carried out at Papworth Hospital in the last 15 years. All surviving patients and medical records of those who died one or more years after transplantation were assessed with respect to skin disease. The clinical data analysis consisted of two major parts:

2.2.1 Retrospective analysis of patient data

Data collection was done by means of

2.2.1.a Patient Questionnaire (Appendix 1)

This was completed by the patient and formed the basis of the initial assessment. The information obtained helped in identifying patients with specific skin problems, in particular, problems such as viral warts, keratoses and skin cancers and gave some indication of potential risk factors including exposure to ultraviolet radiation and family history. Patients during their regular follow-up appointments completed questionnaires. Those who had filled in questionnaires more than two years before the data was analysed or those who had never filled in one were sent out a questionnaire by post to ensure that no skin lesion was omitted. The questionnaires were sent back promptly in most cases. Questionnaires were collected from all surviving transplant recipients.

2.2.1.b Patient records

Detailed data was collected from patient records held at Papworth hospital and supplemented with information obtained on request from the patients' physicians. The other means of data collection was medical notes and the Papworth transplant database. Previously removed skin cancers and keratoses, wherever diagnosed, were reviewed histologically by a histopathologist. Data collection focused on the assessment of the role of various risk factors in causing the skin lesions. These were:

1. Drug history in the context of dose and duration of cyclosporin, prednisolone and azathioprine.
2. Episodes of rejection that needed treatment with anti-rejection drugs such as OKT3, ATG and methyl prednisolone.
3. History of lifetime sun exposure and sun protection.
4. Family history of warts, skin cancers and keratoses.
5. HLA typing of the donor and the recipient.
6. Demographic data such as age, sex and ethnic variations.

2.2.1.c Interview and examination:

Ethical committee approval was obtained prior to the start of the project. Patients were seen individually and further details documented of life-time sun exposure, pre-transplant skin disease, development of skin cancers, warts and pre-cancerous lesions post-transplant. Examination of patients' skin helped in both qualitative and quantitative assessment of skin disorders, at least at one time point for each patient. Information was recorded about skin lesions included type, site and duration. At the same time, any suspect skin lesions were removed. Skin lesions that were removed included mainly warts (common and flat) and keratoses. The skin cancers that

were examined in the study had been removed by dermatologists and plastic surgeons at different places in the country, so paraffin blocks were obtained directly from the patients' histopathologists.

2.2.2 Determination of the role of HPV

2.2.2.a Analysis of diseased tissue

Scrapings or biopsy specimens from all existing and new cutaneous lesions such as warts, keratoses and skin cancers were processed for analysis of the presence and type of HPV using methods described in section 2.1.

Prospective analysis for appearance of HPV

In order to study the temporal acquisition (or rate of activation) and the role in disease of HPV types associated with dysplastic or invasive malignant squamous epithelial lesions, a prospective study of the post-transplant appearance of HPVs in the skin of these immunosuppressed patients was commenced. Scrapings were collected from sun exposed sites in 15 patients at different times post-transplant.

Chapter 3

Results

- 3.1 Laboratory
 - 3.1.1 PCR of paraffin blocks sections
 - 3.1.2 Mini-prep of the positive PCRs
 - 3.1.3 DNA sequencing and sequence analysis
 - 3.1.4 PCR of the frozen tissue normal skin samples
- 3.2 Clinical
 - Statistical Data Analysis
 - 3.2.1 Prevalence data
 - a. Warts
 - b. Keratoses
 - c. Skin cancers
 - 3.2.2 Correlation with risk factors
 - a. Gender
 - b. Age
 - c. HLA associations
 - d. Immunosuppression
 - e. Sun exposure and skin type
 - f. Family history

Results

3.1 Laboratory - DNA analysis from fixed tissue.

3.1.1 PCR of DNA extracted from paraffin blocks sections.

Tissue blocks of skin lesions were obtained and DNA extracted for PCR amplification. Of the 40 samples available, 31 were subjected to a complete DNA analysis. The samples were all between 2-10 years old and the patients were between 5-15 years post-transplant. Histopathological examination by the initial reporting pathologist showed that the lesions were diagnosed variously as squamous cell carcinomas, basal cell carcinomas, Bowen's disease, solar keratoses and Bowenoid actinic keratoses. The DNA from samples the with histology described as SCC, BCC, Bowen's disease, keratoses or Bowenoid actinic keratoses were subjected to a two-step PCR reaction with a nested primer pair to amplify cutaneous HPV DNA. β -globin primers were used as an internal positive control for each of the reactions and to test whether DNA of adequate quality was extracted from the fixed tissue.

The results of the PCR amplification using β globin primers and nested HPV primers are shown in Table 2. For each PCR reaction a positive and a negative control was used. The negative control was wax and the positive control was genomic HPV DNA or a positive sample which had been positive three times. When a positive PCR result was

obtained, the reaction was repeated to a maximum of three times to verify the consistency of the results.

The expected product size in the PCR reaction with the nested primer pair was approximately 470 bp. A positive band in this region present consistently after three PCR attempts was taken as a positive result. The presence of the band once and absence on two occasions was treated as a negative result. The β -globin primers which amplify a 268 base pair region were positive in all the samples which gave positive results with the nested primer pair. There were also positives for some for which DNA was not amplified with the nested primer pair. This is possibly indicative of the fact that although there is some DNA present in the samples it is degenerate and of insufficient quantity to give a positive result with primers which amplify a larger base pair region.

Parameters which affect the PCR reaction were kept in mind and the following precautions were taken :

1. Temperature cycling - Denaturation was done at 95° C for 1 minute, annealing at 56° C for 1 minute and extension at 72°C for 1 minute. These conditions were not altered.
2. MgCl₂ concentration - The concentration of MgCl₂ was optimised at 25mM. Decreasing the concentration below this gave a poor yield of the PCR product.
3. Amount of DNA solution used - Too much of the template DNA solution used may increase the chances of contamination. Since PCR causes greatly increased amplification, a very small amount is needed.

4. dNTP Large amounts of dNTP can inhibit the activity of Taq polymerase enzyme and the concentration of the nucleotides was kept constant at 2Mm.

5. Primer Design - The primers used had already been designed by other groups according to the following set of rules.

- a. The primers were selected to bind in the L1 ORF of the HPV genome, since the L1 is the most conserved region between different HPV types.
- b. The primers were designed be 18-25 nucleotide bases in length. too long a length can result in mismatch and non-specific amplifications.
- c. The ends of the primer should end in a G or a C or GC or CG to increase the efficiency of priming but at the same time more than 3 G or C nucleotides should be avoided to prevent non specific priming.
- d. Primer 3'-ends should not have complementary base pairs to avoid primer-dimer synthesis.
- e. The composition of G and C nucleotides should be about 50% and should be distributed uniformly throughout the full length.

6. Taq Polymerase - More than 1ul of Taq polymerase in a 50µl reaction can cause synthesis of non-specific products

The samples were coded with the first letters of the last name of the patient and the kind of lesion present.

- | | |
|----------------------------|------|
| 1. Squamous cell carcinoma | - S |
| 2. Basal cell carcinoma | - B |
| 3. Bowens disease | - Bo |
| 4. Actinic keratoses | - AK |
| 5. Warts | - W |

6. Bowenoid actinic keratoses - BAK

e.g. John Smith with a squamous cell carcinoma= Sm/S

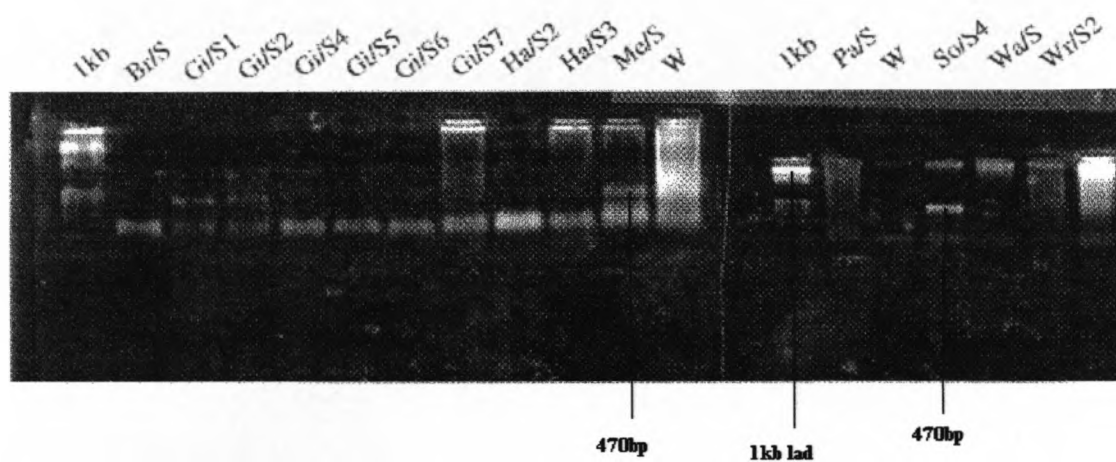
David Jones with 2 squamous cell carcinomas= Jo/S1, Jo/S2

DNA was extracted from a total of 31 lesions from 11 patients. 7 of the patients had only one tissue biopsy, 1 patient had 2, 1 patient had 4, 1 had 5 and 1 had a total of 10 separate lesions. Of the 31 lesions tested, 8 (25%) gave negative results with β -globin primers and also with HPV primers. Of the 22 lesions positive with β -globin primers 16(72%) showed amplification of HPV DNA after the second round of PCR using the nested primer pair. Patients in whom several lesions were tested showed both positive and negative HPV results in different lesions.

Examples of the results of PCR amplification of extracted DNA are shown in Figure 3 (HPV primers) and Figure 4 (β -globin primers)

Fig 3

Results of nested PCR on SCC samples, wax being the negative control and So/S4 the positive control



DNA product of PCR amplification of SCC samples using primers CP 65/70 and CP 66/69A.

PCR reaction of deparaffinated SCC samples with beta-globin primers

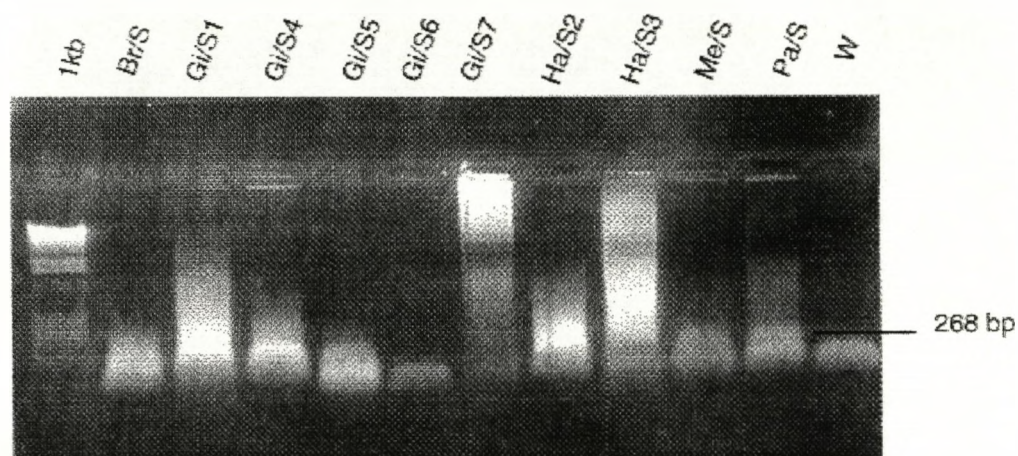


Fig 4

Positive results in the SCC samples with β -globin primers in the 268 bp region. On this occasion, positive PCR product was seen in samples Br/S, Gi/S1, Gi/S4, Gi/S5, Ha/S2, Ha/S3, Mn/S and Pa/S. Gi/S6 and Gi/S7 were negative.

Table 2. Table showing PCR results with nested/ β -glob primers

Patient sample	Age At Tx	Site of lesion	Time post-Transplant (98)	Nested primer pairs	β globin primers
La/S	55	face	6	positive	positive
Me/S	48	ear	9	positive	positive
Ha/S1	54	Face	8	positive	positive
Ha/S2	54	Lip	8	positive	positive
Ha/S3	54	Lip	8	negative	positive
Ha/S4	54	Lip	8	negative	positive
Br/S	53	Face	9	negative	positive
Wr/S	47	Nose	8	negative	negative
So/S1	47	Nose	14	positive	positive
So/S2	47	Ear	14	positive	positive
So/S3	47	Face	14	negative	positive
So/S4	47	face	14	positive	positive
Si/S1	57	Scalp	9	positive	positive
Si/S2	57	Face	9	positive	positive
Si/S3	57	Face	9	negative	positive
Si/S4	57	Face	9	positive	positive
Si/S5	57	Chest	9	negative	positive
Si/S6	57	Face	9	negative	negative
Si/S7	57	Face	9	positive	positive
Si/S8	57	scalp	9	negative	positive
Ca/S	56	Face	11	positive	positive
Ca/S1	47	Ear	7	negative	negative
Ca/S2	47	Leg	7	positive	positive
Ca/A	56	Face	11	negative	negative
Ca/B2	56	Chest	11	positive	positive
Ca/B3	56	Chest	11	negative	negative
Ca/BAK	56	Arm	11	negative	negative
Si/B	57	Dorsum of hand	9	positive	positive

Gi/Bo	57	Leg	9	negative	negative
Sy/B	41	Earlobe	7	negative	negative
Pa/S	49	Face	12	positive	negative

The table depicts the patients last name initials and type of skin lesion present, age at transplant, site of the cancer or keratoses and time post transplant when the data was calculated and paraffin blocks of lesions were worked on. It also shows the PCR results with the nested primer pair and beta-globin primers on paraffin samples.

S-SCC, B-BCC, AK- Actinic keratoses, BAK-Bowenoid actinic keratoses, Bo-Bowens disease.

3.1.2 Mini-prep of the positive PCRs of the paraffin block sections.

DNA was amplified using the nested HPV primers from 16 samples. In each case the PCR products were cloned with the help of a TA cloning kit. Ligation reactions were set up, followed by transformation and then the transformants were cultured overnight. Mini-preps were done for all the positive samples according to the methods listed in Chapter 2 and were checked for DNA yield by an Eco RI digestion. Examples of positive results are seen in Figs 5, 6 and 7.

Fig 5

Mini-preps of samples Gi/S4 and Gi/S7

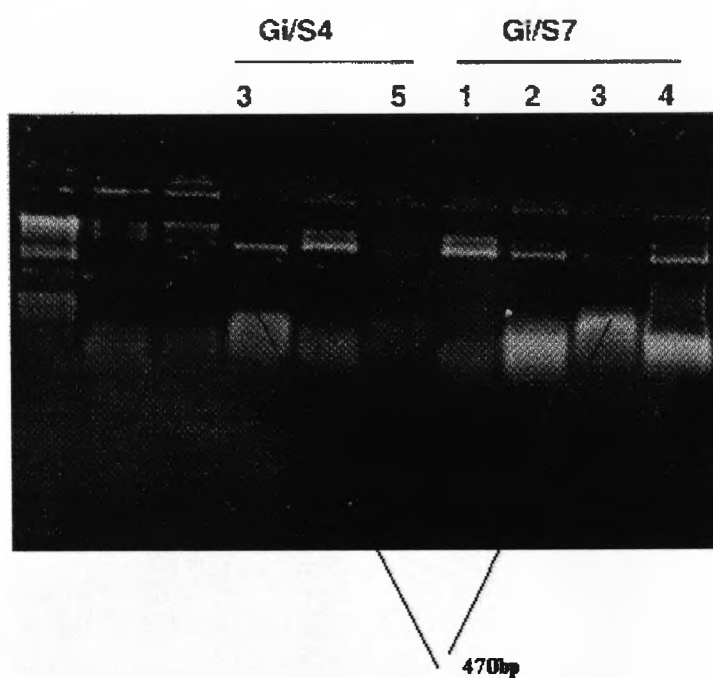


Fig 6

Results of mini-preps of samples CWa/S2 and Ha/S2

CWa/S2 no positive results

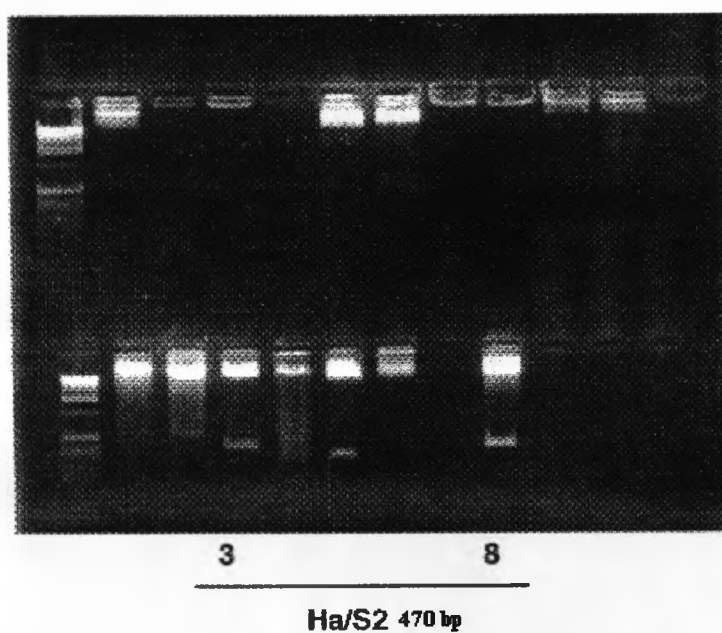
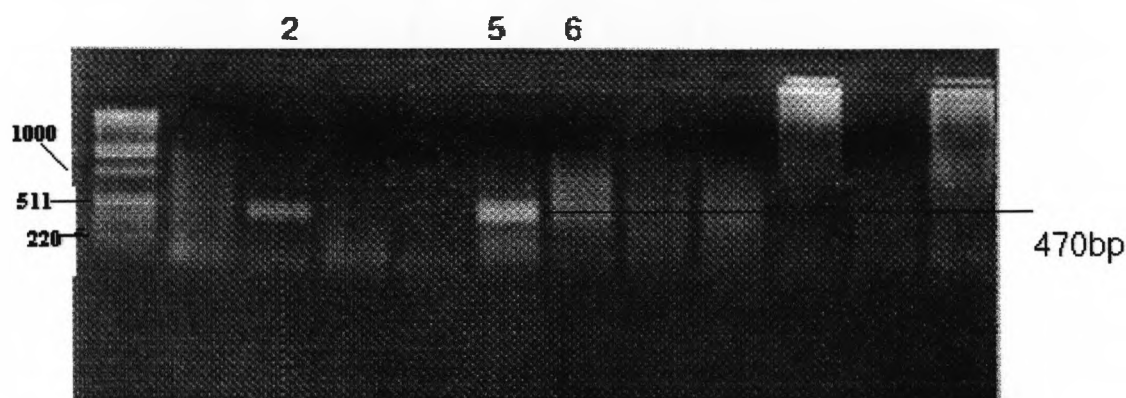


Fig 7**Results of Eco digestion of sample So/S4 after mini-prep**

DNA product of PCR amplification of S0/S4 extracted DNA using primers CP 65/70 and CP 66/69A was cloned into TA cloning vector. Following transformation, DNA was extracted from bacterial colonies and subjected to digestion with Eco R1. DNA was visualised in an agarose gel. The digestion produces a band of 470 base pairs which is visible in clones 2, 5 and 6.

3.1.3 DNA Sequencing and Sequence Analysis

Of the 16 samples positive with PCR, 13 were sent for sequencing to the Department of Biochemistry. In 3 samples, there was insufficient DNA for sequence analysis. Of those sequenced, HPV types were sequenced in 6 samples. No HPV was sequenced in the rest. Interestingly HPV 20 was sequenced in each of the 6 samples. This is an Epidermodysplasia Verruciformis related HPV type (42).

Nucleic acid sequences of the sequenced samples La/S, So/S1, Gi/S7 are illustrated in Figs 8, 9 and 10. The other samples sequenced for HPV 20 were Ha/S2, So/S4 and Gi/S4.

As a result of a search done in Gap package, a high percent identity was found between DNA sequenced from the 6 samples and the L1 ORF of HPV 20 (Fig 11).

HPV 20 is 7757bp in length and was first isolated from skin warts of patients with epidermodysplasia verruciformis and subsequently from a few SCC patients. Even among the EV patients along with EV types 14, 17 and 47, it has been found in few skin cancers as compared with HPV types 5 and 8.

Figure 8.

NUCLEIC ACID SEQUENCE OF La/S

```

1  CTCGAGCGGC CGCCAGTGTG ATGGATATCT GCAGAATTCG GCTTATCAGC
51  TGTTTGTTAC AGTAGTAGAT AATACTCGAA ATACAAATTT TAGCATATCA
101 GTTCATTTCAG AAAACACTGA TGTTTCTAAA ATTCAAAATT ATGACTCTCA
151 GAAATTTCAA GAATATTTAA GACACGTAGA AGAATATGAA ATTTCATTAA
201 TTTTACAGCT CTGTAAAGTT CCTTTAACAG CTGAAGTTTT AGCTCAAATT
251 AATGCTATGA ATTCAAATAT ATTAGAGGAG TGGCAGTTAG GATTCGTTTC
301 TGCACCGGAT AATCCTATCC ACGATACATA CAGATATATT AATTCTGCAG
351 CTACTAGATG TCCTGGTAAA AATCCTCCAA AAGAAAGAGA AGATCCTTAC
401 AAGGATCTAA ACTTCTGGAA TGTAGACATA ACAGAAAGCC GAATTCCAGC
451 ACACTGGCGG CCGTTACTAG TGGATCCGAG CTCGGTACCA AGCTTGATGC
501 ATAGCTTGAG T

```

Figure 9

NUCLEIC ACID SEQUENCE OF S0/S1

```

1  TACCCCNANC TGCCAAAAGG GGGATGTNTT NCAAGCCGAT TAAGTGGGGT
51  ACCGCCAGGG TTTTCCCAGT CACGACGTNG TAAACGACGG CCAGTGAATT
101 GTAATCCGAC TCCACTATAG GCGANTTGGC CNTCTAAATG CATGTTCGAN
151 CGGCCCCGAG TGTGATGAAT ATCTGCAGAA TTCGGGCTTA TCAGCTGTTT
201 GTTNCAGTAG TAGATAATAC TCGAAATNCA AATTTTAGCA TATCAGTTCA
251 TTCAGAAAAC ACTGATGTTT CTAAAATTCA AAATATGAC TCTCAGAAAT
301 TTCAAGAATA TTTAAGACAC GGTAGAAGAA TATGAAATTT CATTAATTTT
351 ACAGCTCTGT AAAGTTCCTT TAACAGCTGA AGTTTTAGCT CAAATTAATG
401 CTATGAATTC AAATATATTA GAGGAGTGGC AGTTAGGATT CGTTTCTGCA
451 CCGGATAATC CTATCCACGA TACATACAGA TATATTAATT CTGCAGCTAC
501 TAGATGTCCT GGTAAAAATC CTCCAAAAGA AAGAGAAGAT CCTTACAAGG
551 ATCTAAACTT CTGGAATGTA GACATAACAG AAAGCCGAAT TCCAGCACAC
601 TGGCGGCCGT TACTAGTGGA TCCGAGCTCG GTACCAAGCT TGATGCATAG
651 CTTGAGTA

```

Fig 10

NUCLEIC ACID SEQUENCE OF Gi/s7

```

1  GCCCAGCTGG CGAAAGGGGG ATGTGCTGCA AGGCGATTAA GTTGGGTAAC
51  GCCAGGGTTT TCCAGTCAC GACGTTGTAA AAACGACGGC CAGTGAATTG
101 TAATACGACT CACTATAGGG CGAATTGGGC CCTCTAGATG CATGCTCGAG
151 CGGCCGCCAG TGTGATGGAT ATCTGCAGAA TTCGGCTTAT CAGCTGTTTG
201 TTACAGTAGT AGATAATACT CGAAATACAA ATTTTAGCAT ATCAGTTCAT
251 TCAGAAAACA CTGATGTTTC TAAAATTCAA AATTATGACT CTCAGAAATT
301 TCAAGAATAT TTAAGACACG TAGAAGAATA TGAAATTTCA TTAATTTTAC
351 AGCTCTGTAA AGTTCCTTTA ACAGCTGAAG TTTTAGCTCA AATTAATGCT
401 ATGAATTCAA ATATATTAGA GGAGTGGCAG TTAGGATTCG TTTCTGCACC
451 GGATAATCCT ATCCACGATA CATACAGATA TATTAATTCT GCAGCTACTA
501 GATGTCTTGG TAAAAATCCT CCAAAGAAA GAGAAGATCC TTACAAGGAT
551 CTAAACTTCT GGAATGTAGA CATAACAGAA AGCCGAATTC CAGCACACTG
601 GCGGCCGTTA CTAGTGGATC CGAGCTCGGT ACCAAGCTTG ATGCATAGCT
651 TGAGTATTCT ATAGTGTAC CTAATAGCT TGGCGTAATC AGGTCATAGC
701 TGTAAGG

```


HPV 20	CTATCCACGATACATACAGATATATTAATTCTGCAGCTACTAGAT
Gi/S7	CTATCCACGATACATACAGATATATTAATTCTGCAGCTACTAGAT
La/S	CTATCCACGATACATACAGATATATTAATTCTGCAGCTACTAGAT
So/S1	CTATCCACGATACATACAGATATATTAATTCTGCAGCTACTAGAT
HPV 20	GTCCTGATAAAAAATCCTCCAAAAGAAAGAGAAGATCCTTACAAGG
Gi/S7	GTCCTGGTAAAAATCCTCCAAAAGAAAGAGAAGATCCTTACAAGG
La/S	GTCCTGGTAAAAATCCTCCAAAAGAAAGAGAAGATCCTTACAAGG
So/S1	GTCCTGGTAAAAATCCTCCAAAAGAAAGAGAAGATCCTTACAAGG
HPV 20	ATCTAAACTTTTGGGAATGTTGACCTATCAGAAA
Gi/S7	ATCTAAACTTCTGGAATGTAGACATAACAGAAA
La/S	ATCTAAACTTCTGGAATGTAGACATAACAGAAA
So/S1	ATCTAAACTTCTGGAATGTAGACATAACAGAAA

3.1.4 PCR of the frozen tissue normal skin samples

At the time of the start of the project, several HPV types had been detected in skin cancers of renal transplant patients and the number reported are rapidly growing. Then, as now, the role of the viral DNA in the development of such tumours was unclear. The carriage rate of the viral DNA in patients had not been specifically shown to increase with time post transplant and it was uncertain if the virus was already present pre-transplant but activated by immunosuppression or whether it was acquired after transplantation when immune resistance was reduced. In order to try to address the question of when such viral DNA could be acquired, we hypothesised that HPV DNA would be detectable on clinically normal skin of immunosuppressed individuals and that the viral load would increase with time post-transplant. If this was the case, HPV DNA should be more readily found in individuals a few years after a transplant, but perhaps not so easily found in individuals early post-transplant. We therefore sought to perform PCR amplification on skin scrapings taken from clinically normal skin of transplant recipients and to analyse such samples in relation to time post-transplant.

DNA was extracted from the following samples which had been collected from the patients, labelled as below and frozen at -70°C and subjected to a phenol/chloroform extraction and ethanol precipitation before PCR amplification.

Different variations of the methods of DNA extraction from tissue and PCR amplification were tried using frozen wart biopsy tissue so as to

optimise the best method for the small tissue samples taken from normal skin. The variations tested included:

1. Varying concentrations of magnesium chloride in the PCR reaction.
2. Using different detergents such as Sodium sarcosine, Triton X 100 and SDS in the proteinase K digestion.
3. Grinding frozen samples with/without dry ice.
4. Addition or omission of sonication of a sample prior to protease digestion.

Results

1. Increasing the quantity of 25mM Magnesium chloride from 1.6µl to 2.0 to 2.4µl increased the amount of DNA amplified.
2. Positive results were obtained with use of detergent Sodium sarcosine and SDS but not with Triton X 100.
3. Samples ground with dry ice gave positive results but not so in the case of the samples which were not ground.
4. Sonicating the sample did not make any difference to the results.

The PCR results from normal tissue are shown in Table 3

Table 3. PCR results of normal skin samples

patient sample Age/Sex	Site collected from	Results of PCR with nested primer pair	Results of PCR with beta globin primers	Results of PCR with Shamanin primers
1 /52 yrs/F	Normal skin / arm	Negative	Negative	Negative
2 /43 yrs/M	Normal skin /hand	Positive	Positive	Positive
3 /54 yrs/M	Normal skin / face	Positive	Positive	Positive
4/58 yrs/F	Normal skin /arm	Positive	Positive	Positive
5/43yrs/M	Normal skin / foot	Positive	Positive	Negative
6/46yrs/M	Normal skin / hand	Negative	Positive	Negative

Normal skin samples were collected from these patients varying from immediately post transplant to 10 years post transplant. The idea was to look for the presence/absence of HPV on normal skin and therefore to determine at what point, if at all HPV is activated on to the skin. It was however not possible to determine the HPV types in the positive samples because of time constraints.

Fig 12.
■

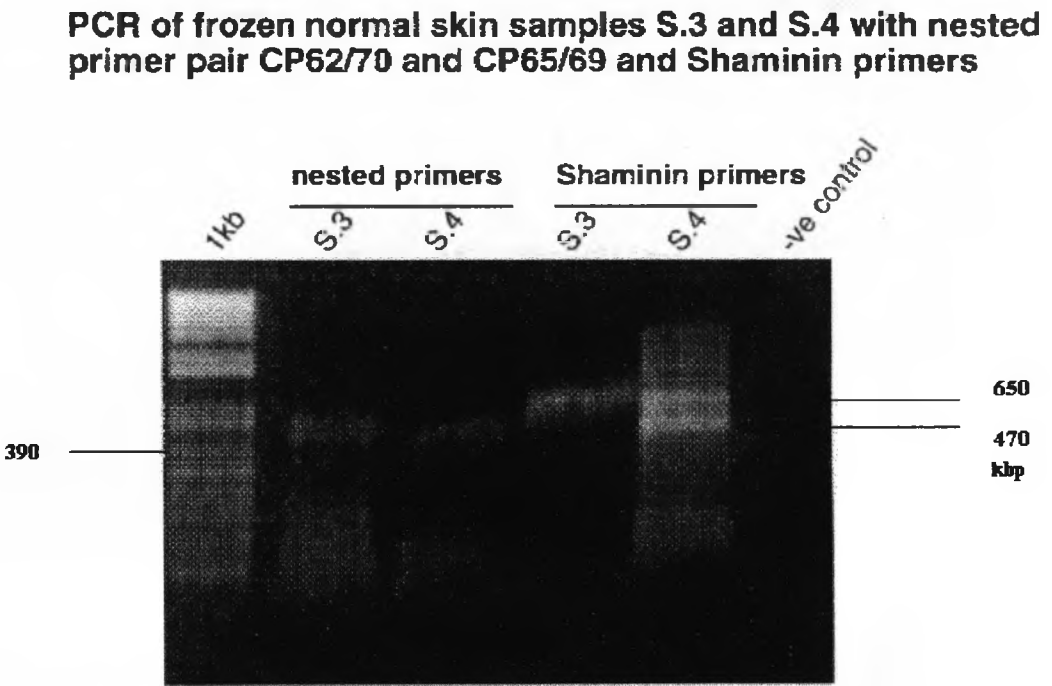


Figure 12 illustrates the example of positive results with PCR, both with the nested primer pair and Shaminin primers. The Shaminin primers amplify a 650 bp region in the L1 ORF. They are degenerate primers designed originally by a German group (72) which identify a wide variety of HPV types. They are suitable for use on fresh and frozen tissue and plasmid DNA only because of the longer length of DNA amplified, which makes them unsuitable for use in paraffin embedded tissue.

The nested primer pair used in this case for normal skin samples was modified in that CP 62 and CP 70 were the outer primer pair and CP65 and CP 69 were the inner primer pair (18). The primer pair is slightly different from the one used for fixed tissue sections with CP 62 being used in place of CP 66.

3.2 Clinical

Statistical Data Analysis

The total number of patients analysed in this study was 397; 296 heart transplant patients, 90 heart-lung transplant patients and 11 lung transplant patients. Both the heart-lung and lung transplant patients were grouped together because they receive similar levels of immunosuppression. They will be referred to as the lung group. All patients were taking corticosteroids and azathioprine. Most, but not all, were also taking cyclosporin.

3.2.1 Prevalence data

Prevalence rates were estimated as the number of patients with the outcome divided by the number who returned questionnaires. 95% confidence intervals were calculated as a measure of the precision of the estimates.

3.2.1.a Warts

- a. The overall prevalence of warts in all groups was 33.8%.
- b. The prevalence of warts in the heart group was 47% (confidence interval 41% to 53 %).
- c. The prevalence of warts in lung transplants was 33% (confidence interval 24 %to 42%).

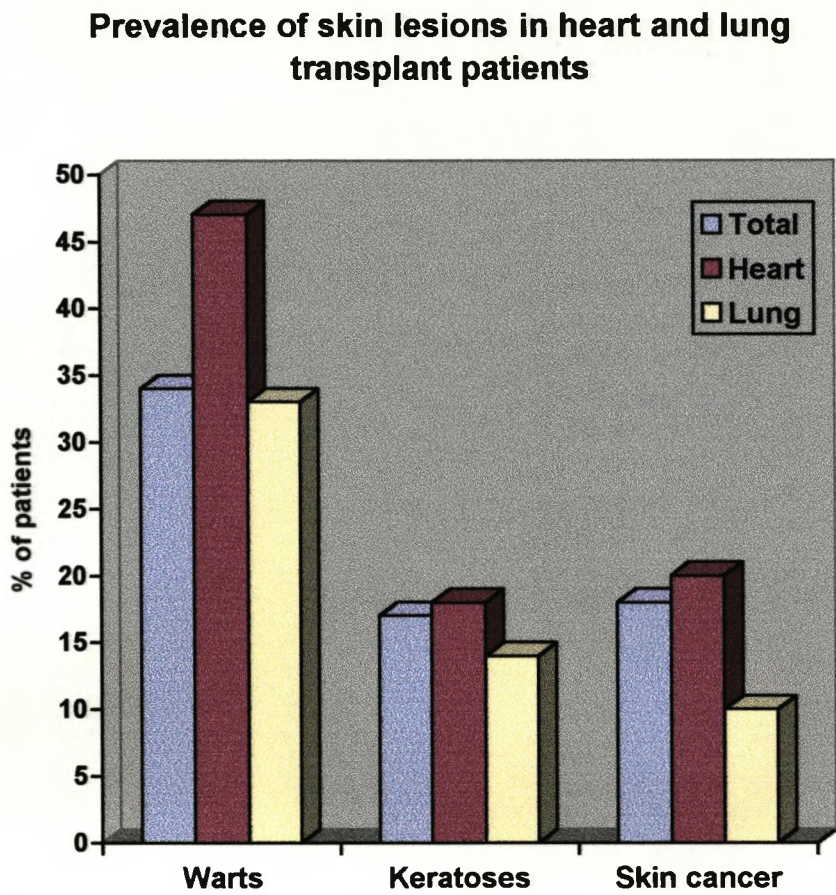
3.2.1.b Keratoses

- a. The overall prevalence of keratoses was 17%.
- b. The prevalence of keratoses in the heart patients was 18% (confidence interval 14% to 22%).
- c. The prevalence of keratoses in lung transplant patients was 14% (confidence interval 7% to 21%).

3.2.1.c Skin cancer

- a. The overall prevalence of skin cancer was 17.6%
- b. Hearts - 20% (15 to 25% confidence interval).
- c. Lungs-10% (confidence interval of 4% to 16%)

Figure 13



The incidence of skin cancer in East Anglia in 1995 was 0.22%. The incidences of the above skin complications could not be calculated because the time that the lesion occurred post transplant was not accurately provided by patients through the questionnaire and also because lesions had been treated in different parts of the country. However it may be expected that the prevalence and incidence are not greatly different, particularly in the case of skin cancers as these are usually treated within a short period of being detected.

When compared with studies on kidney transplant patients the prevalence of skin cancer varied. In a study by Bouwes-Bavink (14) the prevalence of skin cancer was 24.7% (271/1098). In a Spanish study the prevalence was only 1.6% (8/497). This difference could however be because of shorter follow up time periods. Another Spanish study (25) of heart transplant recipients showed a prevalence of 15%, which is within the confidence limits of this study.

3.2.2 Correlation with risk factors

In order to investigate the risk factors for the outcomes, we studied both transplant factors and environmental factors. Environmental factors were age, gender, occupational and social exposure to sunlight, use of sun protection, skin type and hair colour and family history of warts and skin cancer. Transplant factors studied were type of transplant, dose of immunosuppressive therapies and the number of treated rejection episodes in the first 3 months after transplant, the period of greatest immunological onslaught.

Methods used

Initially each categorical risk factor was tabulated against the outcome measures and Pearson's chi-squared statistical test was applied to assess the significance of any associations.

For continuous, normally distributed measurements such as patient age and drug dosages, mean and standard deviations were calculated as summary statistics and Student t-tests were used to assess significance. Where appropriate, the analysis was repeated for heart and lung transplants separately; for example, these groups use different definitions of acute rejection. Univariate analysis however only takes into account one variable at a time.

Finally, multivariate logistic regression was used to determine those variables which were independently associated with the outcomes.

The rationale for multivariate analysis would be to identify the smallest group of factors which would help predict the risk status of patients following cardiothoracic lung transplantation.

Odds ratio and 95% confidence intervals were calculated, whereby the odds on each outcome for a group of patients is compared with the same odds for a baseline group. If the odds ratio is 1 the groups have the same chance of having the outcome. An odds ratio which is significantly greater than 1 indicates that the test group are at greater risk of the outcome than the baseline group. Conversely, an odds ratio of less than 1 means that the test group have a lower risk of the outcome. For example, an odds ratio of 2 indicates that the test group are twice as likely to have the outcome as the baseline group. Logistic regression was done separately for skin cancers, warts and keratoses to look at the association with skin type, sun protection, type of transplant, sex, age and family history as independent variables.

Univariate Analysis

Table 4

Category	Warts	Keratoses	Cancer
Type Of Tx (H)	P=0.013	P=0.35	P=0.021
↑ Age	P=0.304	P=.0002	P=0.002
Sex ↑M	P=0.010	P=0.387	P=0.0007
HLA B27	P=0.838	P=0.504	P=0.755
HLA DR7	P=0.664	P=0.589	P=0.561
HLA A11	P=0.348	P=0.894	P=0.710
Rej	P=0.092	P=0.779	P=0.608
Pred ↓	P=0.031	P=0.708	P=0.648
Aza ↓	P=0.304	P=0.265	P=0.361
CyA ↓	P=0.292	P=0.230	P=0.241
Occup ↑	P=0.089	P=0.205	P=0.212
Social ↑	P=0.333	P=0.324	P=0.015
Sun Protection ↑	P=0.323	P=0.025	P=0.095
Skin types ↓ 1, 2	P=0.266	P=0.030	P=0.072
Hair colour	P=0.757	P=0.446	P=0.659
F/H burns ↑	P=0.003	P=0.778	P=0.858
F/H warts	P=0.019	P=0.470	P=0.297
F/H cancer ↑	P=0.167	P=0.0085	P=0.916

Logistic Regression-Table 5

1. Warts 95% CI

Variables	Exp	Lower	Upper	Result
Type of Tx	1.5479	.8949	2.6775	Heart ↑
Sex	1.8776	1.0319	3.4162	Males↑
Pred	.1697	.0094	3.0650	↑ Pred ↓ risk
F/H Burns	.5739	.3612	.9120	No history ↓ risk
F/H warts	.4905	.2549	.9439	No history ↓ risk

2. Keratoses-Table 6

Variables	Exp	Lower	Upper	Result
Type of Tx	.8250	.3758	1.8114	
Age	1.0772	1.0392	1.1165	↑ age ↑risk
Aza	.7103	.4753	1.0615	
Cyc	1.0456	.9708	1.1262	
Skin type 1	3.0279	.3731	24.5765	
Skin type2	1.8856	.8724	4.0758	
Skin type 3	.8841	.4275	1.8281	
F/H Cancer	.2423	.0919	.6389	No history of Ca ↓ risk

3. Skin cancer-Table 7

Variables	Exp	Lower	Upper	Result
Type of Tx	.8043	.3449	1.8755	
Age	1.0442	1.0125	1.0769	↑ age ↑ risk
Sex	5.3046	1.5218	18.4911	Males ↑ risk
Social exp	.5026	.2581	.9788	No exposure ↓ risk
Protect 1	.4693	.2069	1.0648	
Protect 2	.9266	.4472	1.9198	
Skin type 1	4.1957	.5072	34.7059	
Skin type 2	1.4349	.5806	3.5462	
Skin type 3	1.6696	.8533	3.2669	

3.2.2.a Gender

- a. Warts - Overall 37% of males and 23% females developed warts post transplant. The difference was statistically significant with a p value=0.015.
- b. Keratoses - Overall 17.7% of males and 14% of females transplanted developed keratoses.
- c. Skin cancer - 21.6% of males and only 3.4% of females developed skin cancer post transplant. This reached a statistical significance of $p \leq 0.001$.

3.2.2.b Age

- a. As expected, increasing age was a risk factor for the occurrence of keratoses and skin cancer but not warts.
- b. The difference in the mean ages of patients with skin cancer (48.34) and without skin cancer (42.04) reached a statistical significance of $p=0.0002$.
The difference was similar in patients with keratoses and reached statistically significant levels of $p=0.0002$.

3.2.2.c HLA associations

No positive statistically significant associations were found with HLA B27 or HLA DR7 or negative associations with HLA A11 in the case of warts, keratoses or skin cancers.

3.2.2.d Immunosuppression

- a. Episodes of rejection in the first few months which need treatment with heavy doses of immunosuppressants is one of the markers for immunosuppression. This however was not a risk factor in the occurrence of warts, keratoses or skin cancers in this study.
- b. An unexpected result of the statistical data analysis in the lung transplant group was that patients with lower levels of prednisolone had a higher occurrence of warts and patients receiving lower doses of azathioprine had a somewhat higher prevalence of keratoses.
- c. No other significant associations were seen.

3.2.2.e Sun exposure and skin type

- a. Higher degrees of occupational exposure to the sun had an increased correlation with the occurrence of warts, although this was not statistically significant. In this group the low risk group sun exposure group was used as the baseline group with an odds ratio of 1. The moderate risk group had an increased risk of developing warts by 1.23 times over the baseline group. The high risk sun exposure group had a further 1.23 times increased risk over the moderate risk group. High sun exposure could be a relevant factor in the occurrence of warts in the transplant group although this is not so in the case of the normal population. The patients were grouped into low, moderate and high sun exposure groups depending on the amount of sun they received by virtue of their occupation exposure. There were no other variables included in this risk categorisation

The commonest sites for the occurrence of warts were the dorsum of the hand followed by the face and then the feet. The commonest treatment modality if taken was liquid nitrogen followed by topical therapy (paint) and then curettage and cautery. There was usually good response to treatment with liquid nitrogen. The commonest group of patients however, were those who did not take any sort of treatment. No significant correlation was found between high degrees of occupational sun exposure and increased occurrence of keratoses and skin cancers in the present study.

b. Social exposure to the sun which includes activities such as sun bathing was a definite risk factor in this study for the increased risk of skin cancer. This correlation had a statistical significance of $p=0.015$.

c. As expected, patients with skin types 1 and 2 were more predisposed to the development of keratoses, $p=0.030$, and skin cancer, $p=0.072$. There was, however, no correlation in the case of warts.

d. The commonest skin cancer was SCC followed by BCC and then Bowen's disease. SCCs were mostly present on the face. The commonest site for the occurrence of BCCs was the chest and back. They were usually treated with surgery.

3.2.2.f Family History

a. One of the interesting aspects of the study is that a family history of a tendency to sunburn particularly in Skin types I and II had a significant correlation with the occurrence of warts post-transplant ($p=0.003$)

b. A family history of skin cancer contributed significantly to a risk of keratoses but not skin cancer ($p=0.0085$)

c. As expected, a family history of warts was a risk factor in the occurrence of warts post-transplant ($p=0.019$).

The p value is a probability with value ranging from zero to one. It answers the questions of whether differences observed in different population samples reflects a true difference or is a coincidence of random sampling. If the p value is small, it is possible to conclude that the difference is unlikely to be caused by random sampling. Values > 0.05 are not significant and < 0.001 are extremely significant.

Chapter 4

Discussion and Conclusions

Discussion and conclusions

In this study of heart and lung transplant recipients, we have assessed the size of the problem of long-term skin complications and the potential role of certain risk factors. The skin complications cause morbidity and distress to the patients as well as increasing the dermatological workload on the health service. One of the major aims of this thesis work was to define the risk of skin cancers in heart, heart-lung and lung transplant recipients and to try to evaluate the relative importance of various potential risk factors for cutaneous malignancy in this population.

Using patient questionnaires, examination and molecular analysis of archival material, this study has focused on the development of skin complications and the role of various risk factors such as immunosuppressive regimes, sun exposure, gender, age, ethnic variations and also examined the role played by human papillomaviruses, if any, in causing post-transplant skin cancer in a cohort of heart, heart-lung and lung transplant recipients.

About 1100 patients underwent organ transplantation at Papworth Hospital since 1979. Of the 730 heart, 220 heart-lung and 130 lung transplants carried out at Papworth hospital since 1982, detailed information was obtained from 397. Skin complications such as skin cancer, warts and keratoses were present in a total of 249 (62.7%) patients. Warts were present in 172 patients, skin cancers in 70 and keratoses in 67. This information was gathered from several sources; patient-completed written questionnaires, hospital records and from interview and examination of patients. The most accurate way to

gather such data would be by means of all three methods for each patient. This was not possible for all patients in this population of transplant recipients due to time and geographical constraints.

The development of malignancy is known from other studies to be a significant risk during long-term immunosuppression. In this group of heart and heart-lung transplant patients, skin cancer was the most commonly occurring cancer, and was present overall in 17.6%. The ratio of occurrence of SCC: BCC was 8:1. The ratio in other studies on renal and thoracic organ transplants varies anywhere from equal to 15:1 (6, 7, 25, 34, 39). The median age of patients who had skin cancer was 48 years, which was younger than the 54 years reported in a recent Australian study of heart transplant recipients (69). Although these tumours can cause severe morbidity and some patients needed radical neck dissection and radiotherapy, none of the patients in the present study group died of metastatic skin SCC. This is probably owing to the fact that these patients were followed up well and presented early as they were aware of the complications. In a study (7) from Cincinnati 3 patients out of the 44 patients with epithelial skin cancers developed lymph node metastases and 1 eventually died as a result of skin disease. There is however no evidence to suggest that these patients were not followed up well or that they were not aware of the risks of skin cancer. The inference is that the natural history of skin cancers in the immunosuppressed organ transplant patients is more aggressive than in patients without immunosuppression or transplantation. The historical aspect that people were not aware of skin cancer after transplant 20 years ago, could have influenced the development of cancers in a group of people who have received transplants relatively recently.

The prevalence rate of skin cancers of 20% in the heart transplant patients and 10% in the heart-lung transplant group in this cohort of thoracic transplant recipients was compared with results from the previous studies in kidney and heart transplant patients. In a Norwegian study (41), which looked at 122 heart patients, the period prevalence of skin cancers was 12.9%. A Spanish study (25) of heart transplant recipients showed an incidence of skin cancer of 15.2%. The incidence of skin cancer in a Scottish study (6) of 202 renal transplant patients, 5 years post transplant was 12%. The incidence was less, possibly because of reduced exposure to ultraviolet radiation as compared with places like Spain and Australia. A Dutch study (39) in post renal transplant patients showed an incidence of 10% of skin cancer at 10 years post-transplant and 40% at 20 years post-transplant. An Australian study (38) showed a cumulative risk of 23% at 5 years post renal transplant due to the subtropical locale. The variations in the prevalence and incidence from different studies are probably due to a combination of different factors such as age, different levels of ultraviolet light exposure, variations in the immunosuppressive regimes, race, skin type and the time that the tumour occurred post-transplant.

The present study supported the idea that several factors were interrelated for the development of skin cancers. Ultraviolet light exposure particularly in the form of social activities such as sunbathing, older age at transplant, immunosuppression, skin types 1 (burns easily, tans poorly) and 2 (tans after a little burning) and time post-transplant were all interrelated. The prevalence of skin cancers in heart transplant patients was double that in heart-lung patients mainly because they are in an older age group even though the immunosuppression levels were similar or less. The risk factors above

are in keeping with most other studies, in renal and heart transplant recipients.

47% of heart transplant patients and 33% of heart-lung and lung transplant patients had warts. The prevalence of warts in heart transplant patients in a Norwegian study was 42.6% which was within the confidence limits of this study. Only half of the 70 skin cancer patients had warts and in the same way half of the patients with skin cancer had keratoses. This does not tie in well with the hypothesis postulated by some groups (57) that patients with greater number of warts were at a greater risk of developing SCCs and was not the case in this study. Most of the warts in the present study were on sun exposed sites such as the face, hands and arms. The transformation in the case report (57) from warts to SCCs was on sun-exposed sites, mainly hands and face. Although no such parallel was seen or reported in the present study regular dermatological examinations of warts on sun exposed areas should be carried out.

All the sections analysed by PCR had been examined histologically by the histopathologist at the referring hospital. Since histopathologists do not always make precisely the same diagnosis of tissue biopsies, especially when the disorder might be part of a spectrum of, in this case, epidermal change, it would be of value for the tissue diagnosis to be reviewed by at least one other pathologist.

HPV DNA was detectable in a proportion of archival tissue samples using degenerate primers and was detected in 13 of the 31 samples. Of those 13 samples positive for HPV, 6 gave adequate sequence data and in each case it was HPV 20. The finding of exactly the same type

of HPV in 6 samples raises the doubt that a single contaminant caused the positive results in these 6 samples.

This is despite the fact that all precautions were taken to minimise contamination. This was done by means such cutting sections by using a fresh microtome blade and avoiding repositioning for fresh samples because even that is prone to contamination. In fact, the tissues were scraped by hand by means of a surgical blade for half the paraffin blocks. The fixed tissues are harder than wax and scraping them made the sample more exact. Besides avoiding contamination this also takes care of the problem of normal adjoining tissue and other types of tissue interfering with the polymerase chain reaction. Contamination during the Polymerase chain reaction was kept to a minimum by careful use of the Gilssons and putting blank tubes between normal ones during the PCR run.

Other studies have shown a higher positive rate of HPV detection ranging from 20% to 80% in skin cancers (6, 20, 72) and in normal skin (18) depending on the primers and the techniques used. Our study results are different, and suggest a much lower rate of detection (19%) of HPV DNA despite a much larger number of positive β -globin amplifications which determines the adequacy of the sample to a large extent. This could be because the use of fixed archival tissue makes PCR amplification more difficult. Another reason could be because of the time factor. There was not enough time to try other methods such as using primers which amplify a lower basepair region and verify the results obtained. There are however ways which could improve the detection rate. It is possible that the HPV types were present in low copy numbers, so we could only be certain about their presence and further role in causing skin cancer after designing

primers which amplify a 100-200 basepair length of DNA. It would also be useful to work on frozen tissue samples of skin cancers since DNA in them is not degenerate, thus the chances of detecting HPV DNA are doubled (54). More normal skin samples need to be studied to look for HPV in them, high proportion of virus in normal skin makes it a less likely to be a high risk factor for progression to malignancy. This study was unable to demonstrate either a negative or positive association between skin cancers and Human papillomaviruses. The number of samples analysed were insufficient and even among the positive results we could not be sure if all the samples were HPV 20 since the chances of contamination cannot be ruled out.

The preliminary results of DNA amplification of HPV DNA from scrapings of clinically normal skin in transplant recipients suggested that 4 out of 6 (67%) patients were harbouring viral DNA on the skin surface. Since this study was initiated, there have been several studies reporting the presence of HPV DNA, as detected by PCR, on the skin and in the hair follicles of both immunosuppressed and also, to a lesser degree, immunocompetent individuals(18). Although it was only possible to perform PCR on 6 such patients, our limited results support the concept that HPV DNA is widespread in transplant recipient skin. Given more time, it would be important to sequence the DNA detected to ascertain the specific type involved in these cases. The rate of positivity of the skin scrapings was much higher than the rate of HPV positivity in the tissue biopsies. This may be due to the greater ease of extraction of DNA suitable for PCR amplification from frozen as opposed to fixed tissue. Detection of HPV DNA from skin scrapings or indeed analysis of plucked hairs in transplant patients would give a means of analysing HPV acquisition over time post-transplant. It would also be of interest to know if the HPV types

found on the clinically normal skin of a transplant patient are the same types as those found in the same patient's skin malignancies or pre-malignancies.'

Sun exposure has emerged as a major risk factor as in previous studies (13) and use of sunscreens in this group of transplant patients has not proved particularly beneficial as patients who used more sun protection still had keratoses and skin cancer. This could however be because people who had a tendency to skin cancers and sunburn tended to use more sunprotection. Sunscreens are known to slow the development of keratoses (6) and to reduce sunburn but it is not known to what extent they prevent local immunosuppression which is thought to be a contributory factor in the pathogenesis of skin cancers (76).

Smoking is considered a risk factor in the occurrence of skin cancer (18) but this was not taken into account in this study.

No HLA associations of any sort were found unlike in some previous studies (17) in kidney transplant patients, so presence of HLA-B27 and HLA-DR7 and the presence or absence of HLA-A11 was not considered to confer a protective effect on the occurrence of skin cancer (15). The study had enough power to study HLA associations.

The study is likely to have less than 50% power to detect relationships between HLA mismatches at the A, B and Dr loci and skin lesions. It is very difficult to establish these kinds of relationships in data from a single centre. Relationships between HLA mismatches and mortality have been established from registry data comprising several thousands of transplants but results from individual centres

vary widely. This is because of the following reasons: first, the effect of HLA mismatches are small and modified by many other confounding factors so that large series are required to delineate the effects. Second there is no prospective HLA matching in heart and lung transplantation due to requirement for short ischaemic times, resulting in the vast majority of patients having more than 2 mismatches at these 3 loci.

While the risk of lymphoma is known to be increased by the use of rejection therapies given for episodes of acute rejection particularly OKT3 (65), this factor did not influence the occurrence of skin cancer in the study. Furthermore, no association was found between the occurrence of skin cancers and the other individual immunosuppressant drugs. No relation was found between the occurrence of either warts, keratoses and skin cancers and the number of episodes of rejection that needed treatment.

The study had clinical and statistical limitations. Since the study mode was by means of questionnaires and few interviews and examinations and not always by detailed interviews and examinations, precise details could have been missed which is less likely with patient interaction. Dates of diagnosis of skin complications were not present so only crude point estimates could be done for statistical data analysis. The exact numbers of skin cancers diagnosed for each patient was not known because some patients, particularly ones who had greater number of skin cancers, did not remember the exact number. Measures of immunosuppression were crude and more detailed studies may be needed to make it more sensitive. Given more time, all patients would be questioned directly

and examined, but this was not possible due to time constraints of the study.

Inadequate time was an important constraint in the present study. All the samples obtained could not be analysed.

Future research would focus on designing primers which amplify a smaller basepair region to determine HPV types in the degenerate DNA in archival tissue. We would preferentially like to collect fresh tissue for analysis to increase the chances of HPV detection. Further work on normal skin and hair samples would be useful to determine the passive or active nature of HPV. Another aspect for future research is the immunologic aspects of infection with human papillomaviruses and thus development of a vaccine which could lead to regression of the lesions already present. Antisense oligonucleotides are also being evaluated. The goal of these immunomodulatory agents is not just to treat the cancer but to reduce subclinical and latent virus so that recurrences can be avoided.

Prevention strategies for these complications involve pre-transplant screening of patients, advice regarding avoidance of sun exposure to patients immediately post transplant and retinoid therapy which is thought to prevent the occurrence of keratoses and skin cancer.

Chapter 5

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QUESTIONNAIRE FOR MALE TRANSPLANT RECIPIENTS

Please answer all questions and then return the questionnaire.

Please delete or circle the choices in italics. e.g. *Yes/No* or *Yes/No*

Name.....

Today's Date.....

No.....

DOB.....

Date of Transplant.....

Transplant: *Heart / Heart + Lung / Lung*

Warts

1. Do you have warts? *Yes / No*
2. If yes, where? *Face / Hands / Feet / Arms / Legs / Genital area*
Other site (please specify)
3. Were the warts present before the transplant? *Yes / No*
4. If so, for approximately how long?
5. Have you ever had any treatment for the warts? *Yes / No*
6. If so, what?
6. Did it work? *Yes / No*
7. Did you have warts when you were younger / as a child? *Yes / No*

Keratoses

8. Do you have any keratoses (rough, scaly spots) due to sun damage? *Yes / No*
9. If so, where? *Face / Hands / Feet / Arms / Legs*
Other site (please specify)

Skin Cancer

10. Have you ever had a definite skin cancer? *Yes / No*
11. If so, where? *Face / Hands / Feet / Arms / Legs / Genital area*
Other site (please specify)
12. How was it treated?.....

You and the sun

13. What is your occupation?
14. Have you ever worked regularly out of doors? *Yes / No*
15. If so, what was the work and for how long did you do it?
16. Have you ever lived or worked abroad? *Yes / No*
17. If so, where and for how long.....
18. Do/ did you like to spend time sitting / lying in the sun? *Yes / No*
19. If so, do /did you do this mainly *at home / on holidays abroad?*
20. Do you use a sun protection cream for your skin? *Yes / No*
21. If so, what do you use?.....
22. Do you use this: *Regularly / Occasionally*
23. In the sun, do you: *Burn / Tan after a little burning / Tan but rarely burn*
24. What is your natural hair colour? *Black / Brown / Blond / Sandy / Redhead*

Your family

25. Do your relatives burn in the sun? *Yes / No*
26. If so, please give details.....
27. Have any of your relatives had a skin cancer? *Yes / No*
28. If so, please give details.....
29. Do any of you close relations have warts? *Yes / No*
30. If so, please give details.....

Skin

31. Do you have any other skin trouble? *Yes / No*
If so, please give details.....